Identification of the atypical L-type Ca\(^{2+}\) channel blocker Diltiazem and its metabolites as Ghrelin receptor agonists

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Running Title

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Introduction: 506 words

Discussion: 1023 words

Abbreviations

GHSs: growth hormone secretagogues

GHSR1a: growth hormone secretagogue receptor type 1a
Abstract

Using a high-throughput functional screen, the atypical L-type Ca\(^{2+}\) channel blocker diltiazem was discovered to be an agonist at the human ghrelin (GHSR1a) receptor. In cellular proliferation, Ca\(^{2+}\) mobilization, and bioluminescence resonance energy transfer (BRET-2) assays diltiazem was a partial agonist at GHSR1a receptors, with 50 to 80% relative efficacy compared to the GHSR1a peptide agonist GHRP-6, and high nanomolar to low micromolar potency, depending upon the assay. Seven of the known primary metabolites of diltiazem were synthesized and three of them (M\(_A\), M\(_1\), M\(_2\)) were more efficacious and/or more potent than diltiazem at GHSR1a receptors, with a rank order of agonist activity M\(_2\) > M\(_1\) > M\(_A\) > diltiazem, while M\(_4\) and M\(_6\) metabolites displayed weak agonist activity, and the M\(_8\) and M\(_9\) metabolites were inactive. Binding affinities of diltiazem and these metabolites to GHSR1a receptors followed a similar rank order. In vivo tests showed that diltiazem and M\(_2\) each stimulated growth hormone release in male Sprague-Dawley neonatal rats, though to a lesser degree than GHRP-6. Thus, diltiazem and chemical analogs of diltiazem represent a new class of GHSR1a receptor agonists. The possible contributions of GHSR1a receptor activation to the clinical actions of diltiazem are discussed in the context of the known beneficial cardiovascular effects of ghrelin.
Introduction

Receptors that mediate the effects of growth hormone secretagogues (GHSs), including modified enkephalin peptides (Bowers et al, 1984) as well as small molecules (Smith et al, 1993), each capable of stimulating GH release, were only recently cloned (Howard et al, 1996). Two isoforms (A and B) were identified, but only Growth Hormone Secretagogue Receptor type 1a (GHSR1a) binds and responds to GHSs (Howard et al, 1996). Both isoforms are widely expressed, with the highest levels of GHSR1a found in the arcuate nucleus of the hypothalamus, and lower levels in cardiac tissue (Gnanapavan et al, 2002, Guan et al, 1997). Subsequently, a naturally occurring GHSR1a agonist was identified called ghrelin (Kojima et al, 1999).

A great deal of evidence exists linking ghrelin, GHSR1a receptors, food intake, and obesity in both animal models (Tschop et al, 2000; Nakazato et al, 2001; Asakawa et al, 2003; Shearman et al, 2006) and humans (Wren et al, 2001; Cummings et al, 2001; 2002). However, ghrelin also regulates a variety of other endocrine and metabolic processes including gut motility, energy homeostasis, cellular proliferation, and hormone production (reviewed in van der Lely et al, 2004). Notably, ghrelin, and other GHS ligands produce a number of beneficial effects on cardiovascular function including reductions in vascular resistance, vasodilation, increases in microvascular flow, increases in left ventricular ejection fraction and cardiac output, and cardioprotection in ischemia-reperfusion injury models (Nagaya et al, 2001; 2001b; 2001c; Benso et al, 2004; Cao et al, 2005; Bedendi et al, 2003; Baldanzi et al, 2002). GHSR1a receptors are expressed in heart and vasculature where they may mediate the beneficial effects of ghrelin and GHS.
ligands, though other receptors may also contribute (Baldanzi et al, 2002; Benso et al, 2004; Cao et al, 2005; Bedendi et al, 2003).

Diltiazem, is an L-type Ca^{2+} channel blocker developed over 30 years ago (Uchida, 1976) used to treat patients suffering from angina pectoris, congestive heart failure, chronic obstructive pulmonary disease and coronary artery spasm (Chaffman and Brogden, 1985; Glossman and Striessnig, 1990). Diltiazem exerts its therapeutic effects by reducing vascular resistance, lowering blood pressure, increasing cardiac output, and lowering heart rate. In addition, diltiazem has been shown to provide cardioprotection against ischemia/reperfusion injury in rats (Takeo et al, 2003), similar effects as have been noted for ghrelin and other GHSs (discussed above). Diltiazem is extensively metabolized, and several of these metabolites reach significant concentrations in humans (Molden et al, 2000; Rovei et al, 1980; Sugihara et al, 1984; Sugawara et al, 1988). Several metabolites of diltiazem also show binding activity at Ca^{2+} channels, though all have lower affinity than diltiazem itself (Schoemaker et al, 1987).

To better understand the molecular basis for the clinical actions of drugs, we have been systematically screening and profiling collections of clinically used compounds for activity in functional assays utilizing heterologously expressed G-protein coupled receptors (GPCRs). Using this strategy, diltiazem, and several metabolites of diltiazem were identified as novel GHSR1a agonists in a variety of in vitro and in vivo assays. The possible clinical significance of these findings is discussed.
Methods

**Ligands** - Ghrelin, GHRP-6 (His-DTrp-Ala-Trp-DPhe-Lys-NH2), D-Lys₃ GHRP-6, and substance P analog ([D-Arg1,D-Phe5,D-Trp7,9,Leu11]-substance P) were obtained from American Peptide (Sunnyvale, CA). Verapamil (5-[N-(3,4-Dimethoxyphenylethyl)methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile hydrochloride), Methoxyverapamil, Nifedipine (1,4-Dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester) and Diltiazem (5-(2-(dimethylamino)ethyl)-2-(4-methoxyphenyl)-4-oxo-2,3,4,5-tetrahydrobenzo[b][1,4]thiazepin-3-yl acetate) was purchased from Sigma-RBI. All diltiazem metabolites (MA: 2-(4-methoxyphenyl)-5-(2-(methylamino)ethyl)-4-oxo-2,3,4,5-tetrahydrobenzo[b][1,4]thiazepin-3-yl acetate; M1: 5-(2-(dimethylamino)ethyl)-3-hydroxy-2-(4-methoxyphenyl)-2,3-dihydrobenzo[b][1,4]thiazepin-4(5H)-one; M2: 3-hydroxy-2-(4-methoxyphenyl)-5-(2-(methylamino)ethyl)-2,3-dihydrobenzo[b][1,4]thiazepin-4(5H)-one; M4: 5-(2-(dimethylamino)ethyl)-3-hydroxy-2-(4-hydroxyphenyl)-2,3-dihydrobenzo[b][1,4]thiazepin-4(5H)-one; M6: 3-hydroxy-2-(4-hydroxyphenyl)-5-(2-(methylamino)ethyl)-2,3-dihydrobenzo[b][1,4]thiazepin-4(5H)-one; M8: 5-(2-aminoethyl)-3-hydroxy-2-(4-methoxyphenyl)-2,3-dihydrobenzo[b][1,4]thiazepin-4(5H)-one; M9: 5-(2-aminoethyl)-3-hydroxy-2-(4-hydroxyphenyl)-2,3-dihydrobenzo[b][1,4]thiazepin-4(5H)-one) were synthesized at ACADIA (PCT Publication No. WO/2006/079077).

**Cell culture** – NIH-3T3 cells (ATCC (Manassas, VA) CRL 1658) were incubated at 37 °C in a humidified atmosphere (5% CO₂) in Dulbecco's modified Eagles medium (DMEM) (Invitrogen-Gibco, Carlsbad, CA) supplemented with 25 mM glucose, 4 mM L-
glutamine, 50 U per mL penicillin G, 50 U per mL streptomycin (Invitrogen-Gibco, Carlsbad, CA) and 10% calf serum (Sigma, St Louis, MO) or 25% Ultraculture synthetic supplement (Cambrex, Walkersville, MD). HEK293 (ATCC (Manassas, VA) CRL 1573) cells were cultured similarly except 10% fetal calf serum was substituted for 10% calf serum.

**Constructs.** The GHSR1a receptor used in this study was cloned by polymerase chain reaction using oligonucleotides derived from the GenBank accession entry U60179. Regulator of G-protein signaling 1 (RGS1) was described previously (Burstein et al., 2005). PCRs were performed using Pfu Turbo (Stratagene, La Jolla, CA). All clones were sequence verified before use.

**Cellular proliferation assays** - Cellular proliferation assays (also previously referred to as Receptor Selection and Amplification Technology (R-SAT®) assays were performed as described (Burstein et al., 2006) with the following modifications. Briefly: Cells were plated one day before transfection using 7 x 10³ cells in 0.1 mL of media per well of a 96-well plate (Falcon). Cells were transiently transfected with 5 ng of receptor DNA, 2.5 ng RGS1, and 30 ng pSI-β-galactosidase (Promega, Madison, WI) per well of a 96-well plate using Polyfect (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The use of RGS1 was found to suppress the constitutive activity of GHSR1a and improve agonist responses in this functional assay (Burstein et al., 2005). One day after transfection medium was changed and cells were combined with ligands in DMEM supplemented with 25% Ultraculture synthetic supplement (Cambrex, Walkersville, MD) instead of calf serum to a final volume of 200 µL per well. After five days in culture β-galactosidase levels were measured essentially as described (Burstein et
al, 2005). Cells were rinsed with phosphate buffered saline (PBS), pH = 7.4 before the addition of 200 µL PBS supplemented with 3.5 mM O-nitrophenyl-β-D-galactopyranoside and 0.5% nonidet P-40 (both Sigma, St Louis, MO). After incubation (2-4 h) the plates were read at 420 nm on a plate-reader (Bio-Tek EL 310 or Molecular Devices).

Ca²⁺ mobilization assays - Intracellular changes in calcium concentrations due to activation of GHSR1a receptors were detected using the calcium binding bioluminescence protein aequorin which was expressed as part of a tripartite chimeric protein: MT-GFP-AEQ, as described previously (Burstein et al, 2006).

Bioluminescence Resonance Energy Transfer (BRET-2) assays – BRET assays were performed as described (Schiffer et al, 2006) with the following modifications: HEK293T cells cultured in 10 cm² plates were transiently transfected with plasmid DNAs expressing a bioluminescence donor (1 µg plasmid DNA expressing GHSR1a carboxy-terminally tagged with Renilla luciferase) and a fluorescence acceptor (40 µg plasmid DNA expressing β-arrestin-2 amino-terminally tagged with GFP2. Two days after transfection, cells are harvested and resuspended in phosphate-buffered-saline pH 7.5 (PBS) with glucose and sodium pyruvate to a concentration of 2 x 10⁶ – 4 x 10⁶ cells/mL dependent on transfection efficiency. BRET-2 signals are calculated as the ratio between the Renilla luciferase emission and the GFP2 emission corrected by the background emissions of non-transfected cells.

Binding assays – 15 cm² dishes were seeded with 4 million cells in 16 mL 10% FCS/1% PSG/DMEM for transfection the next day. Plasmid DNA containing the GHSR1a receptor (12.5 µg/dish) in 0.675 mL DMEM was transfected into the cells by
mixing with 180 µL PolyFect, 15 min later mixing in 2.25 mL 10% CS/DMEM, and transfering the mixture into the dish. At 16-18 h post transfection, medium was replaced with 25 mL fresh 10% FCS/1% PSG/DMEM to each dish for another 18-20 h. At approximately 48 hours post-transfection, cells were harvested in ice-cold membrane buffer (20 mM HEPES, 6 mM MgCl₂, 1 mM EDTA, pH to 7.2) using a cell scraper, and pelleted by centrifugation. Pelleted cells were added to a nitrogen cavitation chamber and 900 bar of pressure applied for 30 min. The pressure was slowly released the cavitated cells collected in 50 mL falcon tubes. The tubes were centrifuged at 1000 rpm, 4 °C for 10 min, and the supernatant collected. This centrifugation and collection was repeated two more times until the supernatant was free of precipitate (membranes are still in suspension). The supernatant was poured into a 50 mL centrifuge tube and centrifuged at 10,000 rpm, 4 °C for 20 min. The supernatant was discarded and the pellet resuspended in 750 µL membrane binding buffer using a chilled 1 mL syringe with 25G5/8 needle to re-suspend membranes. The protein concentration was determined using the BioRad Protein Assay Dye Reagent according to the manufacturer’s instructions. The protein concentration was adjusted to 5 mg/mL and aliquots snap-frozen and stored at -80 °C until use. Membranes were thawed rapidly; diluted with binding buffer (25 mM Hepes + 5 mM MgCl₂, 1 mM CaCl₂, 2.5 mM EDTA and 0.2% BSA) to a protein concentration of 0.8 µg/30 µL and placed on ice. 96-well plates (U-bottom wells) were prepared with serial dilutions (8 doses, 40 µL/well) of the test compounds. Membranes (30 µL/well) were then added, and incubated with test ligands for 30 min at room temperature with shaking. 30 µL ¹²⁵I-ghrelin (0.053 nM) was then added to each well, and the plates incubated for another 2.5 hours with shaking. Binding
was terminated by filtration through GF/B filters (presoaked with 0.1% polyethylenimine) with a Brandel 96-well harvester. The filters were washed with ice-cold binding buffer (150 mL/plate) and allowed to air-dry for 30 min. 50 µL MicroScient-20 cocktail was added to each dried well, the plates were sealed, and counted for 2 min/well using a TopCount scintillation counter (Packard).

**Data analysis** – Concentration-response graphs for all functional assays were plotted and EC$\text{_{50}}$ values were determined by nonlinear regression analysis using Prism software (GraphPad version 4.0, San Diego, CA, USA) according to the following equation:

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{\log\text{EC50} - X}}$$

where X is the logarithm of concentration. Y is the response; Y starts at Bottom and goes to Top with a sigmoid shape. Allowing the Hill coefficient to vary did not significantly change the fits of the curves and thus the Hill coefficient was constrained to unity.

**Growth Hormone (GH) releasing assays** – Male Sprague-Dawley neonates (~35 g, 16-day old) were housed with free access to rat chow/water at 6 animals per cage for at least 2 days prior to use. Drugs were dosed i.p. (~9:30am) in 5% DMSO-95% PEG-400 vehicle. Prior to sacrifice, rats were anesthetized with isoflurane (Aerrane, Baxter). Trunk blood samples were collected 15 min after i.p. injection. Plasma was isolated by centrifugation at 3000 rpm for 15 min then stored at -80 °C until use. Rat GH levels were measured using rat growth hormone EIA kits (SPI-BIO, France) according to the manufacturers instructions.
Results

We have developed a high-throughput cellular proliferation assay, which is compatible with most G-protein coupled receptors, and which detects constitutive receptor activity with high sensitivity (R-SAT®, Burstein et al, 2005; 2006). In order to identify novel small molecule ligands, R-SAT® was used to screen the human GHSR1a receptor against a collection of over 200,000 compounds. The compound library included products of combinatorial chemical synthesis, medicinal chemistry synthesis, and over 2000 clinically used drugs. A large number of novel agonists and inverse agonists at the GHSR1a receptor were identified, most of which will not be described further here.

Among the ‘hits’ identified was the L-type Ca²⁺ blocker diltiazem (Figure 1). Full concentration-response experiments confirmed that diltiazem is a partial agonist at GHSR1a, with about 70% efficacy compared with GHRP-6, and a potency of 400 nM (Figure 2, Table 1). Diltiazem-induced responses in cellular proliferation were completely suppressed by the ghrelin-receptor inverse agonist substance P analog, verifying that diltiazem was mediating responses through activation of GHSR1a (data not shown). No significant functional response of GHSR1a receptors was observed to other Ca²⁺ channel blockers verapamil, its methoxy analogue, or nifedipine (Table 1).

Diltiazem is extensively metabolized (Molen et al, 2000; Rovei et al, 1980; Sugihara et al, 1984; Sugawara et al, 1988; see Figure 1), and several of its metabolites also possess Ca²⁺ channel blocking activity (Schoemaker et al, 1987). Seven of the primary metabolites of diltiazem were synthesized and tested for activity at GHSR1a. Several of the diltiazem metabolites were also agonists at GHSR1a. The Mₐ, M₁, and M₂
metabolites were significantly more efficacious, and M\textsubscript{1}, and M\textsubscript{2} were more potent than diltiazem itself, with pEC\textsubscript{50} values of approximately 200 nM (M\textsubscript{1} and M\textsubscript{2}) and efficacies of 89\% to over 100\%, respectively (Figure 2, Table 1). The M\textsubscript{4} and M\textsubscript{6} metabolites displayed weak agonist activity at GHSR1\textalpha. The M\textsubscript{8} and M\textsubscript{9} metabolites did not display agonist activity at GHSR1\textalpha, nor were they able to block GHRP-6 induced responses indicating they did not act as antagonists at GHSR1\textalpha either (data not shown). The potencies of GHRP-6, ghrelin, and hexarelin in the cellular proliferation assay were similar to previously reported values (see Holst et al., 2005), however ghrelin displayed surprisingly low efficacy in the cellular proliferation assay.

GHSR1\textalpha receptors couple the G-protein G\textsubscript{q} to release intracellular Ca\textsuperscript{2+} (Howard et al, 1996), and like virtually all GPCRs, agonist-activated GHSR1\textalpha receptors recruit and bind \beta-arrestin (Holst et al, 2005). Therefore, to verify the results obtained using the cellular proliferation assay, diltiazem and the active metabolites were tested for agonist activity at GHSR1\textalpha in Ca\textsuperscript{2+} mobilization assays, and bioluminescence resonance energy transfer (BRET-2) assays in which luciferase-tagged receptors (GHSR1\textalpha-luc) were co-transfected with GFP-tagged \beta-arrestin-2 (see methods). Separate experiments verified that activity of the GHSR1\textalpha receptor in the cellular proliferation assay was not significantly altered by the presence of the luciferase tag (data not shown). The pEC\textsubscript{50} values of GHRP-6 in the Ca\textsuperscript{2+} and BRET-2 assays were 9.1 and 8.4, respectively, in good agreement with its potency in the cellular proliferation assay (see Table 1). Similar to the cellular proliferation assay, and as seen previously in BRET-2 assays (Holst et al, 2005), ghrelin was a partial agonist in BRET-2, though it was essentially a full agonist in Ca\textsuperscript{2+} mobilization assays.
Testing of diltiazem and its active metabolites in each of these functional assays yielded similar results compared with the cellular proliferation assay (Table 1). In the Ca$^{2+}$ mobilization assay, diltiazem and all the tested diltiazem metabolites were partial agonists, with varying degrees of efficacy, ranging from approximately 54% (diltiazem) to approximately 80% (M2) (Figure 3, Table 1). Potencies ranged from a pEC$_{50}$ of 5.8 for diltiazem to 6.7 for M2. Likewise, in BRET-2 assays, diltiazem and the diltiazem metabolites M$_A$, M$_1$ and M$_2$ were each partial agonists, and again both M$_1$ and M$_2$ were significantly more potent than diltiazem or M$_A$ (Figure 4, Table 1). Thus the rank order of potency across all three functional assays was M$_2$$>$M$_1$$>$M$_A$$>$diltiazem (Table 1).

To directly verify that diltiazem and its primary metabolites are human GHSR1a receptor ligands, radioligand-binding assays were carried out using cell membranes expressing human GHSR1a receptors, using $^{125}$I-ghrelin as the radioligand. Diltiazem, and its primary metabolites M$_A$, M$_1$ and M$_2$ each completely displaced $^{125}$I-ghrelin with a rank order of potency similar to that seen in the functional assays (Table 2).

A characteristic of GHSR1a agonists such as GHRP-6 is the ability to stimulate the release of growth hormone (GH) (Bowers et al, 1984). Diltiazem and the M$_2$ metabolite of diltiazem were administered to male Sprague dawley neonatal rats, and plasma levels of growth hormone assessed. Both diltiazem and M$_2$ were able to stimulate increases in plasma GH levels, though only the increases in response to diltiazem were statistically significant (Figure 5, Table 3). The diltiazem-induced increases were approximately 40% of the maximal effect of GHRP-6.
Discussion

We have used a high-throughput functional screen to identify the L-type Ca\(^{2+}\) channel blocker diltiazem as a human GHSR1a receptor agonist. Using three different functional assays, each measuring distinct functional responses (Ca\(^{2+}\) mobilization, cellular proliferation, and β-arrestin recruitment) we showed that diltiazem is a partial agonist of the human GHSR1a receptor. In addition, we synthesized and tested seven known metabolites of diltiazem, including all of the most abundant ones. Functional profiling revealed that many of these compounds were also agonists at human GHSR1a receptors. Two of the most abundant diltiazem metabolites, M1, and M2, were each more potent, more efficacious GHSR1a receptor agonists than diltiazem itself. The rank order of activity for diltiazem and its metabolites was consistently $M_2 > M_1 > M_A > \text{diltiazem}$ across all functional and binding assays (Table 1, Table 2). In contrast, the rank order of potency of GHRP-6 and ghrelin was ghrelin > GHRP-6 in the cellular proliferation assay, in BRET-2, and in binding assays, but was reversed in Ca\(^{2+}\) mobilization assays, similar to previous observations (Holst et al, 2005). Ghrelin was a partial agonist compared with GHRP-6 in cellular proliferation and BRET-2 assays, in agreement with previous observations that ghrelin is a partial agonist in some functional assays (Holst et al, 2005).

Further tests revealed that diltiazem, and M2, the most potent and efficacious GHSR1a-receptor agonist of the diltiazem metabolites, acted as GHSR1a agonists in vivo. Both diltiazem and M2 stimulated GH-release in neonatal rats. Compared with GHRP-6, the maximal effects of diltiazem and M2 were each approximately 35-40% in GH-release assays, consistent with their partial agonist profiles in vitro.
Diltiazem is used to treat cardiovascular disorders such as angina pectoris, congestive heart failure, chronic obstructive pulmonary disease, hypertension and coronary artery spasm (Abernethy and Schwartz, 1999; Chaffman and Brogden, 1985; Glossman and Striessnig, 1990). Although diltiazem is thought to mediate these effects through blockade of L-type Ca$^{2+}$ channels, it is interesting to speculate whether or not GHSR1a receptors contribute to some of the therapeutic benefits of diltiazem therapy. Peptidyl GHS, non-peptidyl GHS, and ghrelin itself are known to produce a number of beneficial cardiovascular effects including reductions in vascular resistance, vasodilation, increases in microvascular flow, increases in left ventricular ejection fraction and cardiac output, and cardioprotection against ischemia and cellular apoptosis in ischemia-reperfusion injury paradigms (Nagaya et al, 2001; 2001b; 2001c; Benso et al, 2004; Cao et al, 2005; Bedendi et al, 2003; Baldanzi et al, 2002), similar effects as occur with diltiazem administration. Diltiazem has been shown to provide cardioprotection against ischemia/reperfusion injury in rats, and it has been hypothesized that these protective effects are mediated through preservation of mitochondrial function by attenuating Na$^{+}$ overload through Na$^{+}$ channels (Takeo et al, 2003). Possibly the cardioprotective effects of diltiazem in such models may be mediated, in part, through activation of GHSR1a receptors, which have anti-apoptotic effects in cardiomyocytes (Baldanzi et al, 2002). Besides the hypothalamus, GHSR1a receptors are widely distributed, and are expressed throughout the myocardium and vasculature, including ventricle and aorta, (Cao et al, 2005; Gnanapavan et al, 2002, Guan et al, 1997), where they could mediate some of the cardiovascular effects of GHS, ghrelin, and possibly diltiazem. It would be interesting to
evaluate the possible contributions of GHSR1a receptors to the cardioprotective effects of diltiazem using GHSR1a-selective antagonists, or GHSR1a null mice.

Diltiazem has not been reported to elevate GH or cause weight gain clinically (McGraw et al, 1982), and diltiazem was reported to inhibit food intake in Sprague-Dawley rats (Amer and Maher, 2005; de Beaurepaire and Freed, 1989). We have not observed that diltiazem inhibits food intake, and may even modestly stimulate food intake in rats (unpublished observations). The apparent lack of effect chronic diltiazem on GH-release or food intake could be interpreted as a lack of support for the role of ghrelin agonists in these effects, however it is probably more likely due to the primary effects of diltiazem (i.e. blockade of Ca\(^{2+}\) influx), which may impair GH-release (Lussier et al, 1988), and/or inadequate drug exposure (see Rovei et al, 1980; Sugihara et al, 1984; Chaffman and Brogden, 1985) given its potency for activating GHSR1a receptors (this paper) relative to its affinity for L-type Ca\(^{2+}\) channels (Zobrist and Mecca, 1990; Glossmann and Striessnig, 1990), and is also consistent with our observations that diltiazem is a partial agonist at GHSR1a receptors. Diltiazem peak plasma concentrations as high as 152 ng/ml were reported in healthy adult male volunteers (Zelis and Kinney, 1982), comparable to significantly lower than the pEC\(_{50}\) values reported here, depending upon the assay (Table 1). These plasma concentrations are probably insufficient to mediate significant effects on GH-release or food intake given that these are peak rather than sustained concentrations, and do not take into account other factors that would reduce the free concentration of diltiazem at the target tissues such as protein binding (Yamano et al, 2000).
Diltiazem is extensively metabolized (Molden et al, 2000; Rovei et al, 1980; Sugihara et al, 1984; Sugawara et al, 1988), and some of its metabolites, notably desacetyl diltiazem (M₁), reach plasma concentrations approaching 20-30% that of diltiazem itself in humans (Rovei et al, 1980; Sugihara et al, 1984). All of the metabolites studied here displace [3H]-diltiazem binding, allosterically enhance [3H]-nitrendipine binding, and block Ca²⁺-dependent contractions of isolated rat portal veins, but all at lower affinity than diltiazem itself (Schoemaker et al, 1987). The M₂ metabolite, which we found to be significantly more potent and efficacious as a GHSR₁a agonist than diltiazem, had approximately 10-fold lower activity in L-type Ca²⁺ channel binding and functional assays than diltiazem (Schoemaker et al, 1987). Thus the structure-activity relationships for interactions of diltiazem and its metabolites with L-type Ca²⁺ channels and GHSR₁a receptors diverge.

Given the results presented above, medicinal chemistry efforts using the diltiazem scaffold may yield potent GHSR₁a agonists. Indeed, the diltiazem scaffold is amenable to medicinal chemistry, as shown previously (Floyd et al, 1992). The divergence between GHSR₁a activity and L-type Ca²⁺ channel binding activity (Shoemaker et al, 1987) observed in the structural analogs tested here, demonstrates the potential for developing potent GHSR₁a agonists lacking L-type Ca²⁺ channel blocking activity from the diltiazem scaffold.

Acknowledgments

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References


Figure Legends

Figure 1. Diltiazem and its metabolites. Adapted from Sugawara et al., 1988.

Figure 2. Diltiazem pharmacology – cellular proliferation. The human GHSR1a receptor was transfected into NIH3T3 cells and analyzed for proliferative responses as described in the methods. Proliferative responses to the indicated concentrations of A) GHRP-6, diltiazem, M{A}, and B) ghrelin, M{I}, and M{Z}, were measured as described in the methods. Data points are the means of two determinations ± SD.

Figure 3. Diltiazem pharmacology - Ca{sup}2{+} mobilization. The human GHSR1a receptor was transfected with ceolenterazine into HEK293T cells. Cells were harvested and analyzed for Ca{sup}2{+} release in the presence of the indicated ligands as described in the methods. Data points are the means of two determinations ± SD. Ligand responses to GHSR1a were normalized to the response to GHRP-6, which was 40 relative luminescence units (RLUs).

Figure 4. Diltiazem pharmacology - BRET-2. Human GHSR1a receptor fused to renilla luciferase on its C-terminus was co-transfected with human β-arrestin-2 fused to green fluorescent protein (GFP) on its C-terminus into HEK293T cells. Cells were harvested and analyzed for BRET-2 (bioluminescence resonance energy transfer) activity in the presence of the indicated ligands as described in the methods. Data points are the means of two determinations ± SD. Ligand responses to GHSR1a were normalized to their responses to GHRP-6.
Figure 5. Growth Hormone releasing activity of diltiazem and its M₂ metabolite.

Freely moving, freely feeding male Sprague-Dawley rats, approximately 100 to 120 grams, were injected i.p. with the indicated doses (mg/kg) of either diltiazem or the M₂ metabolite of diltiazem. Plasma levels of growth hormone were measured by ELISA as described in the methods. Data are the means of nine to thirty-three determinations ± SEM. (+) indicates 0.5 mg/kg of GHRP-6.
Table 1. Functional profile of Diltiazem and metabolites at the human ghrelin receptor.

Functional assays were performed as described in the methods. Values represent the means +/- SEM. nd indicates not done. nr indicates no response. Me-Verapamil represents methoxyverapamil.

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<tr>
<th>Ligand</th>
<th>Cell proliferation</th>
<th>Ca(^{2+}) mobilization</th>
<th>BRET</th>
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<tr>
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<td>Eff(%)</td>
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<td>GHRP-6</td>
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<td>M4</td>
<td>4.9 +/-0.2</td>
<td>40 +/-6</td>
<td>nd</td>
</tr>
<tr>
<td>M6</td>
<td>4.4 +/-0.4</td>
<td>65 +/-5</td>
<td>nd</td>
</tr>
<tr>
<td>M8</td>
<td>-</td>
<td>12 -</td>
<td>nd</td>
</tr>
<tr>
<td>M9</td>
<td>-</td>
<td>7 -</td>
<td>nd</td>
</tr>
<tr>
<td>Verapamil</td>
<td>-</td>
<td>nr -</td>
<td>nd</td>
</tr>
<tr>
<td>Me-Verapamil</td>
<td>-</td>
<td>nr -</td>
<td>nd</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>-</td>
<td>nr -</td>
<td>nd</td>
</tr>
</tbody>
</table>
Table 2. Binding affinities of Diltiazem and metabolites at the human ghrelin receptor.

Functional assays were performed as described in the methods. Values represent the means +/- SEM.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>pKi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ghrelin</td>
<td>10.4 ± 0.3</td>
</tr>
<tr>
<td>GHRP-6</td>
<td>8.6 ± 0.0</td>
</tr>
<tr>
<td>Hexarelin</td>
<td>8.5 ± 0.0</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>5.5 ± 0.1</td>
</tr>
<tr>
<td>M_A</td>
<td>5.2 ± 0.1</td>
</tr>
<tr>
<td>M_1</td>
<td>6.1 ± 0.2</td>
</tr>
<tr>
<td>M_2</td>
<td>6.3 ± 0.2</td>
</tr>
<tr>
<td>M_4</td>
<td>5.2 ± 0.1</td>
</tr>
<tr>
<td>M_6</td>
<td>4.9 ± 0.0</td>
</tr>
</tbody>
</table>
Table 3. Growth hormone release in rats. Growth hormone releasing assays were performed as described in the methods. Neonatal rats were dosed i.p. with indicated amounts of drugs. 15 minutes post-dosing, animals were sacrificed, blood was harvested, and analyzed for growth hormone levels using a rat growth hormone EIA kit as described in the methods. Data represent means +/- SEM.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg/kg)</th>
<th>GH (ng/ml)</th>
<th>t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>-</td>
<td>10.1 +/- 1.2</td>
<td>-</td>
</tr>
<tr>
<td>GHRP-6</td>
<td>0.25</td>
<td>25.4 +/- 2.2</td>
<td>p&lt; 0.01</td>
</tr>
<tr>
<td>M2</td>
<td>3</td>
<td>14.4 +/- 2.3</td>
<td>p= 0.10</td>
</tr>
<tr>
<td>M2</td>
<td>10</td>
<td>15.3 +/- 2.8</td>
<td>p= 0.08</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>3</td>
<td>14.9 +/- 1.6</td>
<td>p&lt; 0.05</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>10</td>
<td>16.5 +/- 2.7</td>
<td>p&lt; 0.05</td>
</tr>
</tbody>
</table>
Figure 1. Diltiazem and its metabolites

Sagarawa et al. 1988
Figure 2. Cellular proliferation assays
Figure 3. Calcium mobilization assays

- GHRP-6
- Ghrelin
- Diltiazem
- M₁

No Drug

<table>
<thead>
<tr>
<th>% Efficacy</th>
<th>Log [Ligand] M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-11</td>
</tr>
<tr>
<td>25</td>
<td>-10</td>
</tr>
<tr>
<td>50</td>
<td>-9</td>
</tr>
<tr>
<td>75</td>
<td>-8</td>
</tr>
<tr>
<td>100</td>
<td>-7</td>
</tr>
</tbody>
</table>

- GHRP-6
- Mₐ
- M₂

No Drug

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<td>-8</td>
</tr>
<tr>
<td>100</td>
<td>-7</td>
</tr>
</tbody>
</table>
Figure 4. BRET-2 assays

- GHRP-6
- Diltiazem
- M₁
- M₄

- GHRP-6
- M₁
- M₂

Efficacy (%) vs. log [Ligand] M
Figure 5. Growth hormone release

![Growth hormone release graph](image)

GH (ng/ml) vs. Treatment Groups:
- Veh
- M2(3)
- M2(10)
- DTZ(3)
- DTZ(10)

Significance Levels:
- **p<0.01
- *p<0.05

Note: This article has not been copyedited and formatted. The final version may differ from this version.