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Title Page

Growth hormone secretagogues and growth hormone releasing peptides act as orthosteric super-agonists but not allosteric regulators for activation of the G protein $G\alpha_{o1}$ by the ghrelin receptor

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Running title: ligand interactions at the ghrelin receptor

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[1]Abbreviations

AICc, corrected Akaike's Information Criterion; DMEM, Dulbecco's Modification of Eagle's medium; GH, growth hormone; GHRP-6, a hexapeptide growth hormone secretagogue; GPCR, G protein-coupled receptor; [^{35}S]GTPγS, guanosine 5'-*O*-(thio)triphosphate; 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]; MK-677, [N-[1(R)-1, 2-dihydro-1-ethanesulfonylspiro-3H-indole-3,4'-piperidin)-1'-yl]carbonyl-2-(phenylmethoxy)-ethyl-2-amino-2-methylpropanamide]; SPA, [D-Arg^{1},D-Phe^{5},D-Trp^{7,9},Leu^{11}]-substance P.

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ABSTRACT

A series of growth hormone secretagogues act as agonists at the ghrelin receptor and have been described as 'ago-allosteric' ligands due to an ability to also modulate the maximum efficacy and potency of ghrelin (Holst et al., 2005). In membranes prepared from cells co-expressing the human ghrelin receptor and the G protein $G\alpha_{o1}$ each of MK-677, GHRP-6 and L-692,585 functioned as direct agonists, and each displayed higher efficacy than ghrelin. The effect of multiple, fixed concentrations of each of these ligands on the function and concentration-dependence of ghrelin and the effect of multiple, fixed concentrations of ghrelin on the action of MK-677, GHRP-6 and L-692,585 was analyzed globally according to a modified version of an operational model of allosterism which accounts for allosteric modulation of affinity, efficacy and allosteric agonism. Each of the data sets was best fitted by a model of simple competition between a partial and a full agonist. Both positive and negative allosteric modulators are anticipated to alter the kinetics of binding of an orthosteric agonist. However, none of the proposed ago-allosteric regulators tested had any effect on the dissociation kinetics of [His[^{125}I]]-ghrelin and GHRP-6 and MK-677 were able to fully displace [His[^{125}I]]-ghrelin from the receptor. At least in the system tested, each of the ligands acted in a simple competitive fashion with ghrelin as demonstrated by analysis according to a model whereby ghrelin is a partial agonist with respect to each of the synthetic agonists tested.

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INTRODUCTION

The ghrelin receptor (Howard et al., 1996) was identified initially as a regulator of growth hormone (GH[1]) release because it acted as the target of synthetic growth hormone secretagogues that induce stimulation of GH release from the anterior pituitary. The endogenous ligand, ghrelin, is a 28 amino acid peptide cleaved from a 117 amino acid precursor (Van der Lely et al., 2004, Kojima and Kangawa, 2005). As well as key roles produced via ghrelin receptors present on pituitary somatotrophs and on cells in the hypothalamus that trigger release of growth hormone releasing hormone, ghrelin stimulates gastric acid secretion and motility. Furthermore, ghrelin increases food intake, leading to weight gain and reduced fat utilization and circulating ghrelin levels significantly increase during fasting and decrease as a response to food intake (Van der Lely et al., 2004, Leite-Moreira and Soares, 2007). At the same time ghrelin levels are low in obese and high in lean individuals, suggesting that ghrelin is not only important for the acute regulation of food intake but also plays an important role in the regulation of long term energy homoeostasis and, thus, the ghrelin receptor has attracted interest as a potential therapeutic target (Cummings et al., 2005). An intriguing feature of the ghrelin receptor is that it displays a high level of agonist-independent or constitutive activity (Holst et al., 2003) and this appears to be of physiological relevance (Holst and Schwartz, 2006) because mutations that suppress constitutive activity, but not ghrelin-mediated receptor activation, have been associated with both obesity and short stature (Pantel et al., 2006) and hence it would appear that inverse agonism (Milligan, 2003) would be required for a ligand to suppress function of the ghrelin receptor.

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A series of both growth hormone releasing peptides and small molecule growth hormone secretagogues have previously been shown to act as agonists at the ghrelin receptor (Howard et al., 1996, Holst et al., 2005). Moreover, these have recently been described as 'ago-allosteric' ligands at the ghrelin receptor (Holst et al., 2005, Schwartz and Holst, 2006) because, as well as producing direct activation of the receptor, when co-administered with ghrelin such ligands acted to increase the maximum efficacy of ghrelin (Holst et al., 2005). Furthermore, co-administration of ghrelin with ligands including L-692,429 and GHRP-6 either increased or decreased (respectively) the potency of ghrelin (Holst et al., 2005). Thus GHRP-6 and L-692,429 appeared to act both as direct agonists of the ghrelin receptor and as allosteric enhancers or allosteric inhibitors of ghrelin function. Because allosteric modulators are defined as binding to a topographically distinct site than the endogenous ligand (Conn et al., 2009) it is of interest, therefore, that early mutational studies of the ghrelin receptor suggested that the binding sites for GHRP-6, L-692,429 and MK-677 overlap with the binding site for ghrelin (Feighner et al., 1998) and more recent studies from Schwartz and colleagues have confirmed this (Holst et al., 2009).

Measurement of receptor function can be performed at many levels of signal transduction. However, one of the earliest is receptor-mediated activation of a hetero-trimeric G protein. Furthermore, a key feature of allosteric modulators is that they alter the association and/or dissociation kinetics of the binding of orthosteric ligands (Langmead and Christopoulos, 2006). Herein, we use both of these approaches, in combination with data analysis using the operational model of agonist action (Black and Leff, 1983) linked with the allosteric ternary complex model (Ehlert, 1988) to quantify potential allosteric effects on affinity and efficacy as well as allosteric

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agonism (Leach et al., 2007). All the data produced for combinations of growth hormone secretagogues and ghrelin are best described by a simple, competitive binding model but in which ghrelin has lower efficacy to activate the ghrelin receptor than the synthetic ligands.

MATERIALS AND METHODS

Materials Ghrelin and [D-Arg^{1},D-Phe^{5},D-Trp^{7,9},Leu^{11}]-substance P (substance P analog) were purchased from Bachem (St. Helens, Merseyside, UK), GHRP-6 from Sigma-Aldrich (Poole, Dorset, UK), L-692,585 from Tocris (Avonmouth, Bristol, UK) and [His[^{125}I]]-ghrelin from GE Healthcare (Little Chalfont, Buckinghamshire, UK).

Transfections and tissue culture HEK293 cells were grown in Dulbecco's Modification of Eagle's medium (DMEM) supplemented with 10 % (v/v) newborn calf serum and 2 mM L-glutamine. In transient transfection studies cells were transfected with 5 μg/100 cm^2 plasticware of ghrelin receptor cDNA in pcDNA3.1 and/or $G_{\alpha o1}$ in pcDNA3.0 using Lipotectamine (Invitrogen, Paisley, UK) according to the manufacturer's instructions. In all other experiments membranes were prepared from HEK293 cells stably expressing $G_{\alpha o1}$ which were transfected with a ghrelin receptor BacMam at 5 x 10^7 pfu /mL. Sodium butyrate was added to give a final concentration of 2 mM and the transfection incubated for 24 h at 37°C.

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[^{35}S]GTPγS Binding Assays [^{35}S] guanosine 5'-*O*-(thio)triphosphate (GTPγS) binding experiments were performed using two separate methods. In Figure 1 cell membranes (10 μg) were incubated in 900 μl of buffer (20 mM HEPES, 100 mM NaCl, 6 mM $MgCl_2$, 0.1 % BSA; pH 7.4) containing 10 μM GDP, 0.1 nM [^{35}S]GTPγS and varying concentrations of ligands. The reaction was incubated at 30°C for 20 min and subsequently terminated by rapid filtration through GF/C filters using a Brandel cell harvester (Brandel, Gaithersberg, MD), filters washed three times with 3 mL of ice-cold phosphate-buffered saline and bound radioactivity determined by liquid scintillation counting. All other experiments used a [^{35}S]GTPγS scintillation proximity assay where membranes were resuspended in assay buffer (20 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl, 0.05 % (v/v) bovine serum albumin (BSA) and 0.05 % (v/v) pluronic F-127; pH 7.4 at 25°C) to a concentration of 50 μg/mL and preincubated with 8 μM GDP and 2 mg/mL of wheat germ agglutinin polystyrene LEADseeker imaging beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK) under gentle agitation for 30 min (25 °C). 25 μL of this mixture and 25 μL of a final concentration of 0.6 nM [^{35}S]GTPγS diluted in assay buffer were added to each well of a 384-well white plate stamped with 0.5 μL ligand, centrifuged (800 x *g*; 2 min) and, after 80 min incubation, bound [^{35}S]GTPγS determined by scintillation counting. In experiments designed to examine potential interaction between two compounds, the assay was performed with the compound that was to be kept at a fixed concentration (e.g. ghrelin for Figure 3 and MK-677, GHRP-6 or L-692,585 for Figure 4) mixed under gentle agitation for 30 min (25 ºC) with 50 μg/mL membranes (resuspended in assay buffer as previously detailed), 8 μM GDP and 2 mg/mL of wheat germ agglutinin polystyrene

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LEADseeker imaging beads. 25 μL of this mixture and 25 μL of a final concentration of 0.6 nM [^{35}S]GTPγS diluted in assay buffer were added to each well of a 384-well white plate stamped with 0.5 μL of either MK-677, GHRP-6 or L-692,585 (Figure 3) or ghrelin (Figure 4), centrifuged (800 x *g*; 2 min) and, after 80 min incubation, bound [^{35}S]GTPγS determined by scintillation counting.

[His[^{125}I]]-ghrelin binding assays Cell membranes (5 μg) were incubated in triplicate with a final concentration of 83 pM [His[^{125}I]]-ghrelin in a final volume of 150 μL of assay buffer (50 mM Tris-base, 2 mM EGTA, 0.1 % (w/v) BSA; pH 7.3 at 4 ºC) (Muccioli et al, 2001). Non-specific binding was determined by the inclusion of 1 μM ghrelin. Reactions were incubated for 120 min at 4 ºC and terminated by rapid filtration through GF/B filters pre-soaked in 0.5 % (w/v) polyethyleneimine (PEI) and washed three times with 1 mL ice-cold assay buffer. Bound [His[^{125}I]]-ghrelin was measured by liquid scintillation counting.

[His[^{125}I]]-ghrelin competition binding assays To establish whether the growth hormone secretagogues could compete with [His[^{125}I]]-ghrelin for binding to the ghrelin receptor, various concentrations of GHRP-6, L-692,585 and MK-677 were added to the assay mix and the experiment initiated, terminated and measured as described above.

[His[^{125}I]]-ghrelin dissociation assays For dissociation experiments, after binding for 120 min at 4 ºC, 1 μM ghrelin, ± varying concentrations of GHRP-6, L-692,585 or

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MK-677 were added to prevent re-association of [His[^{125}I]]-ghrelin to the ghrelin receptor following dissociation.

Data analysis Data analysis was performed using GraphPad Prism software (versions 4.0 and 5.0; GraphPad Software Inc., San Diego, CA). Unless otherwise stated, concentration-response curve data were analyzed according to a four parameter logistic fit with data points representing the mean ± standard error of the mean of three independent experiments performed in triplicate. Agonist concentration-response curves, in the absence and presence of SPA, were globally fitted to the following logistic equation (Eq. 1; Motulsky and Christopoulos, 2004):

$$Y = Bottom + \frac{(Top - Bottom)}{1 + \left(\frac{10^{\log EC_{50}} \left[1 + \left([B] / 10^{-pA_2} \right)^s \right]}{[A]} \right)^{n_H}} \quad (1)$$

where *Top* represents the maximal asymptote of the curves, *Bottom* represents the lowest asymptote (basal response) of the curves, $LogEC_{50}$ represents the logarithm of the agonist EC_{50} in the absence of antagonist, *[A]* represents the concentration of the agonist, *[B]* represents the concentration of the antagonist, n_H represents the Hill slope of the agonist curve, *s* represents the Schild slope for the antagonist, and pA_2 represents the negative logarithm of the concentration of antagonist that shifts the agonist EC_{50} by a factor of two. If the estimated Schild slope was not significantly different from unity, it was constrained as such and the estimate of pA_2 represented the pK_B.

To investigate whether the interaction between the partial agonist, ghrelin, and the

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higher efficacy agonists (GHRP-6, MK-677 or L-692,585) is allosteric or simply competitive, a more complex model which incorporates the agonist activity of both compounds under test is required. The [^{35}S]GTPγS binding datasets studying the effect of multiple, fixed concentrations of ghrelin on concentration-response curves to GHRP-6, MK-677 or L-692,585 were analyzed globally according to a modified version of an operational model of allosterism which accounts for allosteric modulation of affinity, efficacy and allosteric agonism (Leach et al., 2007). The equation represents a simplified model whereby it is assumed that the concentration-response curve data is to a full agonist (Eq. 2):

$$Y = Basal + \frac{(E_M - Basal)\cdot(([A](K_B + \alpha\beta[B])) + \tau_B[B]\cdot EC_{50})^n}{(([A](K_B + \alpha\beta[B])) + \tau_B[B]\cdot EC_{50})^n + (EC_{50}{}^n \cdot (K_B[B])^n)} \quad (2)$$

where *Basal* is the response in the absence of ligand, EC_{50} is the midpoint of the full agonist concentration-response curve, K_B is the equilibrium dissociation constant of the putative allosteric ligand, τ_B denotes the capacity of the putative allosteric ligand to exhibit agonism (a function of the intrinsic efficacy and receptor expression) and $\alpha\beta$ represents a net affinity / efficacy co-operativity parameter which describes the effect of the putative allosteric ligand on agonist function. The terms E_M and n denote the maximal possible system response and the slope factor of the transducer function that links occupancy to response, respectively. In all fits the latter value was constrained to 1.

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If the interaction between ghrelin and GHRP-6, MK-677 or L-692,585 was competitive, then the value of $\alpha.\beta$ would be zero (because the value of the affinity cooperativity factor, α, would be zero) and Eq. 2 would reduce to that for the interaction of a partial agonist and full agonist binding to the same site. Therefore, the datasets were analyzed under two conditions – where the value of $\alpha\beta$ is left to float or constrained to zero. Comparisons of the two fits were performed using Akaike's Information Criterion (AICc; Motulsky and Christopoulos, 2004) to determine which fit was most likely to be correct.

In order to further validate the results of the interaction studies, experiments were performed to study the effects of multiple, fixed concentrations of GHRP-6, MK-677 or L-692,585 on concentration-response curves to ghrelin. These data were analyzed using a re-cast version of Eq. 2 such that the concentration of ghrelin is the independent variable on the x-axis (i.e. full agonist vs partial agonist). As previously, the datasets were analyzed under two conditions (where the value of $\alpha\beta$ is left to float or constrained to zero) and the fits compared using AICc.

RESULTS

The ghrelin receptor is most widely recognized as a G protein-coupled receptor (GPCR) able to couple effectively to the phosphoinositidase C-linked $G\alpha_q/G\alpha_{11}$ family G proteins and hence to the elevation of intracellular Ca^{2+} levels (Howard et al., 1996, Holst et al., 2003, 2005, van der Lely et al., 2004). However, as with many other GPCRs (Gudermann et al., 1996, Wise et al., 1997) it is also able to modulate cellular signalling via pathways initiated via activation of other hetero-trimeric G proteins (Holst et al., 2005, Camiña et al., 2007, Dezaki et al., 2007). When

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membranes of HEK293 cells transfected transiently to express both the ghrelin receptor and the G protein $G\alpha_{o1}$, were employed in [^{35}S]GTPγS binding studies, substantial levels of bound [^{35}S]GTPγS were recovered in the absence of addition of ligands (**Figure 1**). This was not observed in membranes of equivalent cells transfected to express $G\alpha_{o1}$ but not the ghrelin receptor (**Figure 1**) and is consistent with the ghrelin receptor displaying significant constitutive capacity to activate $G\alpha_{o1}$. Addition of a single, maximally effective concentration of ghrelin (1×10^{-6} M) was without effect in the absence of the ghrelin receptor but produced a significant, approximately 2 fold, increase above basal levels of bound [^{35}S]GTPγS in membranes expressing both $G\alpha_{o1}$ and the ghrelin receptor (**Figure 1**). Further indication of the constitutive capacity of the ghrelin receptor to active $G\alpha_{o1}$ was that a substance P analog [D-Arg1,D-Phe5,D-Trp7,9,Leu11]Substance P (1×10^{-6} M), previously described as a ghrelin receptor inverse agonist (Holst et al., 2003, 2005) was able to reduce basal levels of [^{35}S]GTPγS binding substantially in membranes co-expressing $G\alpha_{o1}$ and the ghrelin receptor (**Figure 1**).

A series of both growth hormone releasing peptides, e.g. GHRP-6 and small molecule growth hormone secretagogues, including MK-677, and L-692,585, have previously been shown to act as agonists at the ghrelin receptor. Each of these ligands, as well as ghrelin, increased binding of [^{35}S]GTPγS in a concentration-dependent manner in membranes of HEK293 cells stably expressing $G\alpha_{o1}$ and transfected to express the ghrelin receptor transiently (**Figure 2**). Compared to ghrelin, each of these three ligands was a 'super-agonist', generating maximal efficacy (E_{MAX}) greater than ghrelin, whilst GHRP-6 and L-692,585 also acted with significantly lower potencies than ghrelin (see **Table 1** for potency and efficacy

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values). To explore these observations and the suggestion that a number of synthetic agonist ligands also act as allosteric regulators of the action of ghrelin and hence as 'ago-allosteric' ligands (Holst et al., 2005), a series of [^{35}S]GTPγS binding studies was performed on membranes of HEK293 cells co-expressing $G\alpha_{o1}$ and the ghrelin receptor. In these multiple, fixed concentrations of ghrelin were added and concentration-response curves to each of MK-677, GHRP-6, and L-692,585 were then performed. For MK-677, the presence of ghrelin at concentrations ranging from 1 x 10^{-11} M to 3 x 10^{-10} M, which stimulated [^{35}S]GTPγS binding to between 10 and 50% of the level that could be achieved by a maximally effective concentration of MK-677, did not alter the potency or E_{MAX} of MK-677 (**Figure 3A; Table 2A**). At ghrelin concentration of 1x 10^{-9}M and 1 x 10^{-7} M a significant reduction in potency of MK-677 was observed (**Table 2A**), whilst, only in the presence of 1 x 10^{-7}M ghrelin was the E_{MAX} of MK-677 decreased (**Table 2A**). To determine whether the interaction between ghrelin and the secretagogues was likely to be allosteric or merely competitive, analysis of the data was performed using a modified version of an operational model of allosterism (Leach et al., 2007; see Methods). Comparison of data fits using Akaike's Information Criterion (Motulsky and Christopoulos, 2004) showed a clear preference for the simpler model with the value of $\alpha.\beta$ constrained to zero (**Figure 3A, Supplementary Table S1**). Therefore, the [^{35}S]GTPγS binding studies with ghrelin and MK-677 do not provide evidence to favor an allosteric mode of interaction between the two ligands but instead favor a competitive model in which a partial agonist (ghrelin) and a full agonist (MK-677) bind to a common site (see **Discussion** for further details). In equivalent experiments employing GHRP-6 or

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L-692,585 (**Figures 3B, 3C**), concentrations of ghrelin up to 3 x 10^{-10} M again did not alter the potency or E_{MAX} of these ligands **Table 2B, 2C**). Similar to MK-677, only at a concentration of 1 x 10^{-7}M ghrelin was there a reduction in E_{MAX} of L-692,585, whilst varying concentrations of ghrelin did not alter the E_{MAX} of GHRP-6 (**Table 2C**). Comparison of data fits using Akaike's Information Criterion (**Supplementary Table S1)** again showed a clear preference for the simpler model with the value of $\alpha.\beta$ constrained to zero, hence all the data were consistent with ghrelin acting at a site that can be considered to be orthosteric with the synthetic compounds tested.

To explore this further the experimental protocol was reversed and the effect of multiple, fixed concentrations of the synthetic compounds on concentration-response curves to ghrelin was assessed. At 3 x 10^{-11} M MK-677 the effect of increasing concentrations of ghrelin was still to increase binding of [^{35}S]GTPγS above the level produced by MK-677 (**Figure 4A; Table 3A**). However, due to the 'super-agonist' effect of MK-677 compared to ghrelin, at all concentrations of MK-677 at and above 1 x 10^{-9} M increasing concentrations of ghrelin caused a decrease in [^{35}S]GTPγS binding (**Figure 4A**). Comparison of the data fits was performed using the same model as described above, but re-cast such that the partial agonist, ghrelin, was the independent variable on the x-axis. As would be expected, the estimates for parameters such as the pEC_{50} of MK-677 and affinity of ghrelin were similar to the previous estimates, despite the reversed protocol (**Supplementary Table S1**). As previously, the comparison of the data fits using AICc suggested that interaction between ghrelin and MK-677 was likely to be competitive (see **Discussion**).

Entirely equivalent data was obtained for ghrelin concentration-response curves performed in the presence of varying concentrations of GHRP-6 (**Figure 4B;**

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Table 3; Supplementary Table 1) and L-692,585 (**Figure 4C; Table 3; Supplementary Table 1**). The data for both of these compounds fitted better to a competitive, rather than allosteric model, once more consistent with ghrelin sharing the orthosteric binding site with each of these three ligands.

Both MK-677, GHRP-6 and, less potently, L-692,585 (**Figure 5**) were able to compete with [^{125}I]ghrelin and limit its specific binding. Although sufficiently high concentrations of L-692,585 could not be employed in these studies to assess this directly, both MK-677 and GHRP-6 were able to compete fully with [^{125}I]ghrelin and in a monophasic manner (**Figure 5**), again consistent with these ligands competing for a common binding site (**Table 4**). Analysis of the L-692,585 inhibition curve (constraining minimum to zero and using K_d = 250 pM (see below) and [^{125}I]ghrelin = 83 pM) result in an estimated pK_i = 5.6 for L-692,585. Such competition binding studies do not, however, provide clear insight into the mechanism of the reduction in specific [^{125}I] ghrelin by these ligands. Allosteric ligands are predicted to alter the kinetics of binding of orthosteric agonists (Langmead and Christopoulos, 2006), an effect that is often monitored by measuring changes in dissociation of a radiolabelled orthosteric ligand. In membranes of HEK293 cells co-expressing $G\alpha_{o1}$ and the ghrelin receptor, association of [^{125}I]ghrelin to specific binding sites was fitted adequately by a monophasic hyperbola and reached a plateau within 120 minutes when incubated at 4^{o}C (**Figure 6A**). Dissociation studies were initiated by the addition of 1 x 10^{-6} M ghrelin following an initial 120 minute incubation to allow binding of [^{125}I]ghrelin. Under these conditions dissociation of [^{125}I]ghrelin was monophasic (**Figure 6B**) and the measured K_{obs} and K_{off} values resulted in an estimate for K_d of 2.53 x 10^{-10} M for [^{125}I]ghrelin. To test potential allosteric effects directly [^{125}I] ghrelin dissociation

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studies were performed in the presence of L-692,585. This had no effect on the kinetics of [^{125}I] ghrelin dissociation (**Figure 7A, 7B**). With estimated $pK_i = 5.6$, 1 μM L-692,585 would only be predicted to occupy some 30% of receptors. However, this is the highest concentration of ligand that we could employ for these studies. However, various concentrations of either MK-677 or GHRP-6, consistent with substantially higher receptor occupancy, also failed to alter the rate of dissociation of [^{125}I] ghrelin (**Figure 7B**). These data are again consistent with lack of an allosteric effect of these ligands on the binding of [^{125}I]ghrelin.

In studies exploring the effect of the substance P analog on ghrelin concentration-response curves for stimulation of [^{35}S]GTPγS binding in membranes of HEK293 cells co-expressing $G\alpha_{o1}$ and the ghrelin receptor, increasing concentrations of the substance P analog caused a progressive rightward shift in the EC_{50} for ghrelin to higher concentrations. However, an associated reduction in apparent ghrelin E_{MAX} was observed and such an effect could be consistent with a non-competitive mechanism of inhibition. However, as shown in **Figure 1** the substance P analog acts as an inverse agonist for ghrelin receptor activation of $G\alpha_{o1}$ and, therefore, basal binding of [^{35}S]GTPγS in the absence of ghrelin was reduced by the presence of the substance P analog (**Figure 1**). When the inverse agonist effect of the substance P analog was accounted for, increasing concentrations of the substance P analog produced parallel and surmountable rightward shifts in the concentration-response to ghrelin (**Figure 8A; Supplementary Table S2**) that resulted in Schild plots with slope value not significantly different from 1.0 (0.81 +/- 0.18) and an estimated pK_B for the substance P analog of 6.58 ± 0.18. Similar data was obtained when varying concentrations of the substance P analog were used to explore the

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effectiveness of MK-677 ($pK_B = 7.10 \pm 0.10$), GHRP-6 ($pK_B = 7.49 \pm 0.09$) and L-692,585 ($pK_B = 7.45 \pm 0.10$) to stimulate binding of [^{35}S]GTPγS (**Figure 8B,C,D**). Fitting the basal data to a concentration-response curve revealed that the potency of the substance P analog for reducing the constitutive activity of the receptor was similar to the pK_B values obtained from the Schild regression data and furthermore, revealed that maximally effective concentrations of the substance P analog could reduce the constitutive activity of the ghrelin receptor to a level 54.1 ± 3.2 % of that measured in the absence of inverse agonist (**Supplementary Figure 1**).

DISCUSSION

A series of both non-peptide growth hormone secretagogues and synthetic growth hormone releasing peptides are known agonists of the ghrelin receptor**.** Previous studies exploring the effects of a number of these on both the binding of [^{125}I]ghrelin and the function of ghrelin in COS-7 cells transfected to express the human ghrelin receptor have indicated inconsistencies in their action in different assay end points and shown them to possess characteristics of allosteric regulators of the action of ghrelin (Holst et al., 2005). Such data have resulted in the generation of a complex model that evokes the necessity of the ghrelin receptor existing as a dimer and in which the various positive and negative allosteric effects on the action of ghrelin may be explained by the growth hormone secretagogues and growth hormone releasing peptides binding in distinct ways to the individual protomers of the ghrelin receptor dimer (Holst et al., 2005, Schwartz and Holst, 2006). This is intriguing because there are a growing number of instances in which ligands with highly

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selective affinity and/or potency for one GPCR can affect the pharmacology, function and/or cellular distribution of a second GPCR for which they have no inherent direct affinity if the two GPCRs form a hetero-dimer (El-Asmar et al., 2005, Ellis et al., 2006, Parenty et. al., 2008) and this has been discussed in terms of allosteric effects across the hetero-dimer interface (Milligan and Smith, 2007). However, such effects are substantially more challenging to explore for potential GPCR homo-dimers unless a mutated receptor, designed to alter its affinity to pharmacological agents, is paired with the corresponding wild type receptor to generate an asymmetric homo-dimer or pseudo hetero-dimer that has distinct pharmacology at each protomer (Damian et al., 2006, Sartania et al., 2007).

It is now becoming obvious that many, and perhaps all, GPCRs are able to regulate a range of intracellular signals and there is considerable interest in the concept of different agonists being able to selectively modulate one or other pathway (Kenakin, 1995). Such 'biased' ligands may offer therapeutic advantage (Urban et al., 2007, Michel and Alewijnse, 2007). Along with the well characterized activation of $G\alpha_q/G\alpha_{11}$ family G proteins that results in elevation of intracellular Ca^{2+} levels (Howard et al, 1996, Holst et al., 2005), activation of the ghrelin receptor has been reported to generate signals mediated via the stimulatory G protein $G\alpha_s$ (Malagon et al., 2003) and pertussis toxin-sensitive G proteins of the G_i-family (Dezaki et al., 2007). There is also great interest in the prospect that 'allosteric' ligands which bind to a distinct site on the receptor than the endogenous 'orthosteric' ligand, may be able to generate selective effects at individual subtypes of closely related receptors that share a common orthosteric ligand, for example the muscarinic acetylcholine receptors (Christopoulos et al., 1998).

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In the model used herein, the human ghrelin receptor and the pertussis toxin-sensitive G protein $G\alpha_{o1}$ were co-expressed in HEK293 cells. A pair of well studied growth hormone secretagogues and a growth hormone releasing peptide each acted as super-agonists in activating $G\alpha_{o1}$ compared to ghrelin. However, analysis of the datasets according to a modified version of an operational model of allosteric interaction (Leach et al., 2007; see Methods) provided no evidence to support either positive or negative allosteric effects of the various ligands studied on the action of ghrelin. Instead such analysis favored a simpler model in which ligands of distinct efficacy compete at the orthosteric site. Allosteric ligands can cause a change in the location of an agonist concentration-response curve. This is usually manifest as a rightward or leftward shift, dependent on the nature of the co-operativity between the agonist and allosteric ligand. Allosteric co-operativity has historically been only considered in terms of effects on ligand affinity, denoted by the parameter, α, which is a bi-directional, thermodynamic measure of the ratio of affinities of the orthosteric ligand in the presence and absence of the allosteric ligand, Values of $\alpha > 1$ represent positive co-operativity (and increase in agonist affinity and hence potency) whereas values of $\alpha < 1$ represent negative co-operativity (and a decrease in affinity and hence potency). A value of $\alpha = 1$ represents neutral co-operativity whereby the allosteric ligand does not alter agonist affinity. At very low values ($\alpha < 0.01$) a negatively cooperative interaction becomes almost indistinguishable from that of simple competition (where $\alpha = 0$). It is now recognised that in addition to effects on affinity, allosteric ligands can modulate agonist efficacy and even activate receptors in their own right (Langmead and Christopoulos, 2006). From a practical perspective, a number of models have been developed to analyze datasets displaying such a range of

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behaviors. These models utilize the operational model of agonist action (Black and Leff, 1983) combined with the allosteric ternary complex model (Ehlert, 1988) to quantify the allosteric effects on affinity and efficacy as well as allosteric agonism (Leach et al., 2007).

One of the hallmarks of an allosteric interaction is that any effects on agonist affinity and / or efficacy, whether positive or negative, are saturable and reflect the degree of co-operativity between the two ligands. This is in contrast to the effects of a competitive antagonist which is theoretically limitless in its effect on agonist function. Relatively low concentrations of ghrelin had no effect on the location of the agonist curves produced by GHRP-6, MK-677 and L-692,585, but caused increases in [^{35}S]GTPγS binding in its own right. At 1 x 10^{-7} M ghrelin caused a rightward shift in the concentration-response curve to all three synthetic agonists consistent with a competitive mode of action. However, ghrelin appears as a high efficacy partial agonist with respect to all three agonists and as such the window with which to examine the mechanism of interaction using this assay design is limited. In order to better profile the mechanism of action of the synthetic agonists, reverse studies were performed to examine the effects of multiple, fixed concentrations of GHRP-6, MK-677 or L-692,585 on a concentration response curve to ghrelin. In the absence of synthetic agonist, ghrelin stimulated [^{35}S]GTPγS binding in a concentration-dependent manner. Increasing concentrations of GHRP-6, MK-677 or L-692,585 also stimulated [^{35}S]GTPγS binding, but to a level over and above the maximal ghrelin response. At the highest concentrations of the synthetic agonist, increasing concentrations of ghrelin actually inhibit [^{35}S]GTPγS binding to the same level as the maximal response to ghrelin in the absence of synthetic agonist. Analysis of the

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datasets according to the operational model described in the Methods showed a clear favor for a competitive fit in preference to an allosteric mechanism of interaction.

These studies do not attempt to replicate the model system used by Holst and colleagues (2005) and thus do not inherently repudiate their conclusions on the ago-allosteric actions of at the ghrelin receptor of growth hormone secretagogues and growth hormone releasing peptides. However, these data in combination with the ligand dissociation rate studies provide clear evidence that, at least for direct activation of $G\alpha_{o1}$ by the human ghrelin receptor, all three synthetic agonists examined share the orthosteric site with the endogenous ligand, ghrelin. Early studies indicated an overlapping binding site for ghrelin with many of these ligands, based on the similar effect on a Glu3.33 mutation in transmembrane domain III of the receptor (Feighner et al., 1998) and this is certainly also consistent with orthosteric and competitive actions of each ligand. Furthermore, recent mutational studies from Holst and colleagues have provided further evidence for the overlap of binding sites of the endogenous agonist ghrelin with growth hormone secretagogues and growth hormone releasing peptides (Holst et al., 2009). Clearly the nature of the orthosteric binding site in receptors with large peptide ligands poses a substantial challenge for pharmacological definition of the mode of action of synthetic agonist ligands Equally, these studies do not attempt to explore whether the ghrelin receptor acts as a dimer as suggested by the ago-allosteric model (Schwartz and Holst, 2006). Although there are now a number of reports that indicate that purified and reconstituted GPCR monomers can cause activation of G proteins (Whorton et al., 2007, 2008) there is a general consensus that many GPCRs do exist as dimers and/or higher order oligomers (Milligan, 2007, 2008), although the specific relevance of this for pharmacology and

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function remains a highly active area of research and debate. The current data highlight the contribution pharmacological modelling can provide to understanding and the need to apply 'Occam's razor' to analysis of data sets.

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Footnotes

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Figure Legends

Figure 1 The ghrelin receptor is able to cause constitutive activation of $G\alpha_{o1}$: substance P analog is an inverse agonist

HEK293 cells were transfected to express $G\alpha_{o1}$ (**open bars**) or $G\alpha_{o1}$ and the ghrelin receptor (**filled bars**). The binding of [^{35}S]GTPγS in membranes of these cells in the absence of ligand or the presence of ghrelin or substance P analog (SPA) (both 1 μM) was then assessed . Data are presented as the % of the effect of ghrelin in membranes co-expressing $G\alpha_{o1}$ and the ghrelin receptor (means +/- SEM, n = 3). ***p<0.001, **p<0.01 (one-way ANOVA with Tukey's multiple comparison test).

Figure 2 A number of growth hormone secretagogues and growth hormone releasing peptides act as super-agonists for ghrelin receptor-mediated activation of $G\alpha_{o1}$

The ability of varying concentrations of ghrelin, GHRP-6, MK-677, and L-692,585 (as indicated) to enhance binding of [^{35}S]GTPγS in membranes of HEK293 cells transfected to co-express $G\alpha_{o1}$ and the ghrelin receptor was assessed. Data points represent means +/- SEM of 4 independent experiments performed in triplicate. See **Table 1** for quantitative details.

Figure 3 Ghrelin does not alter the E_{MAX} of MK-677, GHRP-6 or L-692,585 to activate $G\alpha_{o1}$ via the ghrelin receptor

[^{35}S]GTPγS binding experiments were performed on membranes of HEK293 cells transfected to co-express $G\alpha_{o1}$ and the ghrelin receptor. Data points represent the mean ± S.E.M of 3 independent experiments performed in triplicate. Data were fitted

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to Equation 2, data shown fitted with $\alpha\beta=0$ and the slope transducer function constrained to 1.

A. A series of concentration-response curves to MK-677 was performed in the absence (control) or presence of varying concentrations of ghrelin (as indicated).

B. Equivalent studies were performed with GHRP-6 and varying concentrations of ghrelin

C. Equivalent studies were performed with L-692 585 and varying concentrations of ghrelin

Figure 4 Growth hormone secretagogues and growth hormone releasing peptides do not alter the E_{max} of ghrelin to activate of $G\alpha_{o1}$ via the ghrelin receptor

[^{35}S]GTPγS binding experiments were performed on membranes of HEK293 cells transfected to co-express $G\alpha_{o1}$ and the ghrelin receptor. Data points represent the mean ± S.E.M of three independent experiments performed in triplicate. Data were fitted to Equation 2, data shown fitted with $\alpha\beta=0$ and the slope transducer function constrained to 1.

A. A series of concentration-response curves to ghrelin was performed in the absence (control) or presence of varying concentrations of MK-677 (as indicated)

B. Equivalent studies were performed with ghrelin and varying concentrations of GHRP-6

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C. Equivalent studies were performed with ghrelin and varying concentrations of L-692 585

Figure 5 The specific binding of [^{125}I] ghrelin is inhibited by the presence of growth hormone secretagogues and growth hormone releasing peptides

The specific binding at 4^{o}C of [^{125}I]ghrelin to membranes of HEK293 cells co-expressing Gα_{o1} and the ghrelin receptor was measured over a 120 minute period in the absence and presence of varying concentrations of ghrelin, GHRP-6, MK-677 or L-692,585 (as indicated). Data points represent means +/- SEM of 3-5 independent experiments. Data are fitted to a one-site competition model.

Figure 6 [^{125}I]Ghrelin binds to and dissociates from the ghrelin receptor in a monophasic fashion

A. The specific binding at 4^{o}C of [^{125}I]ghrelin to membranes of HEK293 cells co-expressing Gα_{o1} and the ghrelin receptor was measured over time. Data were fitted to a monophasic hyperbola consistent with K_{obs} = 0.029 min^{-1}. Data points represent mean ± S.E.M of three independent experiments performed in triplicate.

B. Following association of [^{125}I] ghrelin as above for 120 minutes, dissociation of the ligand was measured over time following addition of 1 x 10^{-6}M ghrelin. Data are presented as a semi-log plot. K_{off} = 0.02 +/- 0.00 min^{-1}. Data points represent the mean of three independent experiments performed in triplicate.

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Figure 7 Growth hormone secretagogues and growth hormone releasing peptides do not affect the dissociation of [^{125}I] ghrelin

As in Figure 6B, the loss of specific binding at 4^{o}C of [^{125}I]ghrelin to membranes of HEK293 cells co-expressing $G\alpha_{o1}$ and the ghrelin receptor was assessed over time as a measure of the dissociation rate.

A. As well as addition of 1 x 10^{-6}M ghrelin at time 0, L-692,585 (3 x 10^{-7}M) was also present. K_{off} = 0.01 +/- 0.00 min^{-1}. Data points represent mean ± S.E.M of three independent experiments performed in triplicate.

B. Varying concentrations of GHRP-6 (upper panel), L-692,585 (middle panel) or MK-677 (lower panels) were added along with 1 x 10^{-6}M ghrelin. The level of specific binding of [^{125}I]ghrelin was then measured at time 0 and at 60 minutes. Data points represent mean ± S.E.M of three independent experiments performed in triplicate, data shown as semi-log plots and analyzed using linear regression.

Figure 8 Substance P analog is a competitive antagonist of the agonist actions of ghrelin, GHRP-6, L-692,585 and MK-677.

Concentration-response curves were generated to A. ghrelin B. GHRP-6, C. L-692,585 or D. MK-677 in the presence of multiple, fixed concentrations of SPA. Data shown normalized with 0 % equal to the basal [^{35}S]GTPγS binding obtained in the presence of SPA. Data were fitted to Equation 2, with the Schild slopes and Hill slopes shared across the data sets (see Supplemental Table 2). Data points represent the mean ± S.E.M of three individual experiments performed in triplicate (black = no substance P analog; red = 30 nM substance P analog; blue = 0.1 μM substance P

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analog; green = 0.3 μM substance P analog; purple = 1 μM substance P analog; orange = 3 μM substance P analog; pink = 10 μM substance P analog).

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Tables

Table 1 – Potency and efficacy of ghrelin and the growth hormone secretagogues as measured using a [^{35}S]GTPγS scintillation proximity assay. *p<0.05, **p<0.01 as measured using a one-way ANOVA with Dunnett's post-hoc test to compare the potencies and efficacies of GHRP-6, MK-677 and L-692,585 to that of ghrelin. E_{MAX} is the maximum efficacy of each ligand, where 100 % equals the maximum efficacy of ghrelin. Data were fitted with concentration-response curves with Hill slopes constrained to 1.

Ligand	pEC_{50} ± S.E.M	E_{MAX} ± S.E.M
Ghrelin	9.11 ± 0.10	95.4 ± 3.4
GHRP-6	7.85 ± 0.13**	139.5 ± 5.4*
MK-677	9.21 ± 0.12	139.6 ± 9.9*
L-692,585	7.60 ± 0.17**	145.4 ± 14.0*

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Table 2 - Potency and efficacy of A. MK-677, B. L-692,585 and C. GHRP-6 in the presence of increasing concentrations of ghrelin, as measured using a [^{35}S]GTPγS scintillation proximity assay. Data were fitted with concentration-response curves with the Hill slope shared between datasets. The Hill slopes were **A.** 0.78 ± 0.10 , **B.** 0.87 ± 0.10 and **C.** 0.80 ± 0.09. *p<0.05, **p<0.01 measured using a one-way ANOVA with Dunnett's post-hoc test. E_{MAX} displayed as % of maximum response to each ligand in the absence of ghrelin.

A

Condition	pEC50 ± S.E.M	EMAX ± S.E.M
MK-677	9.58 ± 0.03	99.7 ± 0.8
+ 10 pM ghrelin	9.43 ± 0.03	105.1 ± 0.9
+ 0.1 nM ghrelin	9.27 ± 0.06	96.8 ± 1.3
+ 0.3 nM ghrelin	9.16 ± 0.13	93.7 ± 2.6
+ 1 nM ghrelin	8.32 ± 0.10**	107.1 ± 1.5
+ 100 nM ghrelin	7.82 ± 0.21**	94.6 ± 2.3*

B

Condition	pEC_{50} ± S.E.M	E_{MAX} ± S.E.M
L-692,585	7.77 ± 0.03	101.3 ± 1.4
+ 10 pM ghrelin	7.65 ± 0.03	111.4 ± 1.7
+ 0.1 nM ghrelin	7.65 ± 0.05	102.3 ± 2.0
+ 0.3 nM ghrelin	7.50 ± 0.13	102.0 ± 4.4
+ 1 nM ghrelin	6.80 ± 0.12**	111.5 ± 3.0

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+ 100 nM ghrelin	7.84 ± 0.25	90.3 ± 1.6*

C

Condition	pEC_{50} ± S.E.M	E_{MAX} ± S.E.M
GHRP-6	8.55 ± 0.02	99.6 ± 1.0
+ 10 pM ghrelin	8.41 ± 0.05	110.6 ± 1.9
+ 0.1 nM ghrelin	8.40 ± 0.70	106.5 ± 2.4
+ 0.3 nM ghrelin	8.17 ± 0.13	105.0 ± 3.8
+ 1 nM ghrelin	7.31 ± 0.22**	106.5 ± 4.0
+ 100 nM ghrelin	7.55 ± 0.35**	93.4 ± 2.8

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Table 3 - Potency and efficacy of ghrelin in the presence of increasing concentrations of A. MK-677, B. L-692,585 and C. GHRP-6 as measured using a [^{35}S]GTPγS scintillation proximity assay. Data were fitted with concentration-response curves with the Hill slope shared between datasets. The Hill slopes were for **A.** 1.07 ± 0.31, **B.** 1.14 ± 0.28 and **C.** 1. *p<0.05, **p<0.01 measured using a one-way ANOVA with Dunnett's post-hoc test. E_{MAX} displayed as % of maximum response of ghrelin achieved in the absence of the growth hormone secretagogues.

A

Condition	pEC_{50} ± S.E.M	E_{MAX} ± S.E.M
Ghrelin only	8.54 ± 0.02	96.0 ± 0.8
+ 0.03 nM MK-677	7.84 ± 0.06*	117.2 ± 1.8**
+ 0.1 nM MK-677	8.03 ± 0.31	121.5 ± 6.6**
+ 1 nM MK-677	9.07 ± 0.12	159.0 ± 1.7**
+ 3 nM MK-677	8.50 ± 0.07	190.8 ± 1.3**

B

Condition	pEC_{50} ± S.E.M	E_{MAX} ± S.E.M
Ghrelin only	8.54 ± 0.05	97.3 ± 1.8
+ 3 nM L-692,585	8.03 ± 0.27	111.7 ± 7.7
+ 10 nM L-692,585	Not fitted	Not fitted
+ 30 nM L-692,585	9.53 ± 0.78	129.8 ± 10.6*
+ 1 μM L-692,585	7.80 ± 0.39	177.9 ± 5.6**

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C

Condition	pEC_{50} ± S.E.M	E_{MAX} ± S.E.M
Ghrelin only	8.64 ± 0.02	100.2 ± 0.7
+ 0.1 nM GHRP-6	8.32 ± 0.21*	108.3 ± 7.6
+ 1 nM GHRP-6	8.01 ± 0.08	103.1 ± 1.5
+ 10 nM GHRP-6	9.01 ± 0.15	134.3 ± 2.2**
+ 100 nM GHRP-6	7.67 ± 0.15**	187.6 ± 2.5**

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Table 4 – pK_i and Hill slope values obtained for ghrelin, MK-677, GHRP-6 and L-692,585 competing with [^{125}I]-ghrelin binding to the ghrelin receptor. The use of an F-test revealed data were best fitted to one-site competition curves. In each instance the Hill slope obtained was not significantly different from unity.

Ligand	$pK_i \pm$ S.E.M	Hill slope ± S.E.M
Ghrelin	8.97 ± 0.27	0.59 ± 0.41
GHRP-6	7.51 ± 0.71	0.52 ± 0.63
MK-677	8.14 ± 0.08	0.67 ± 0.49
L-692,585	< 6.00	Not fitted

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Figure 1

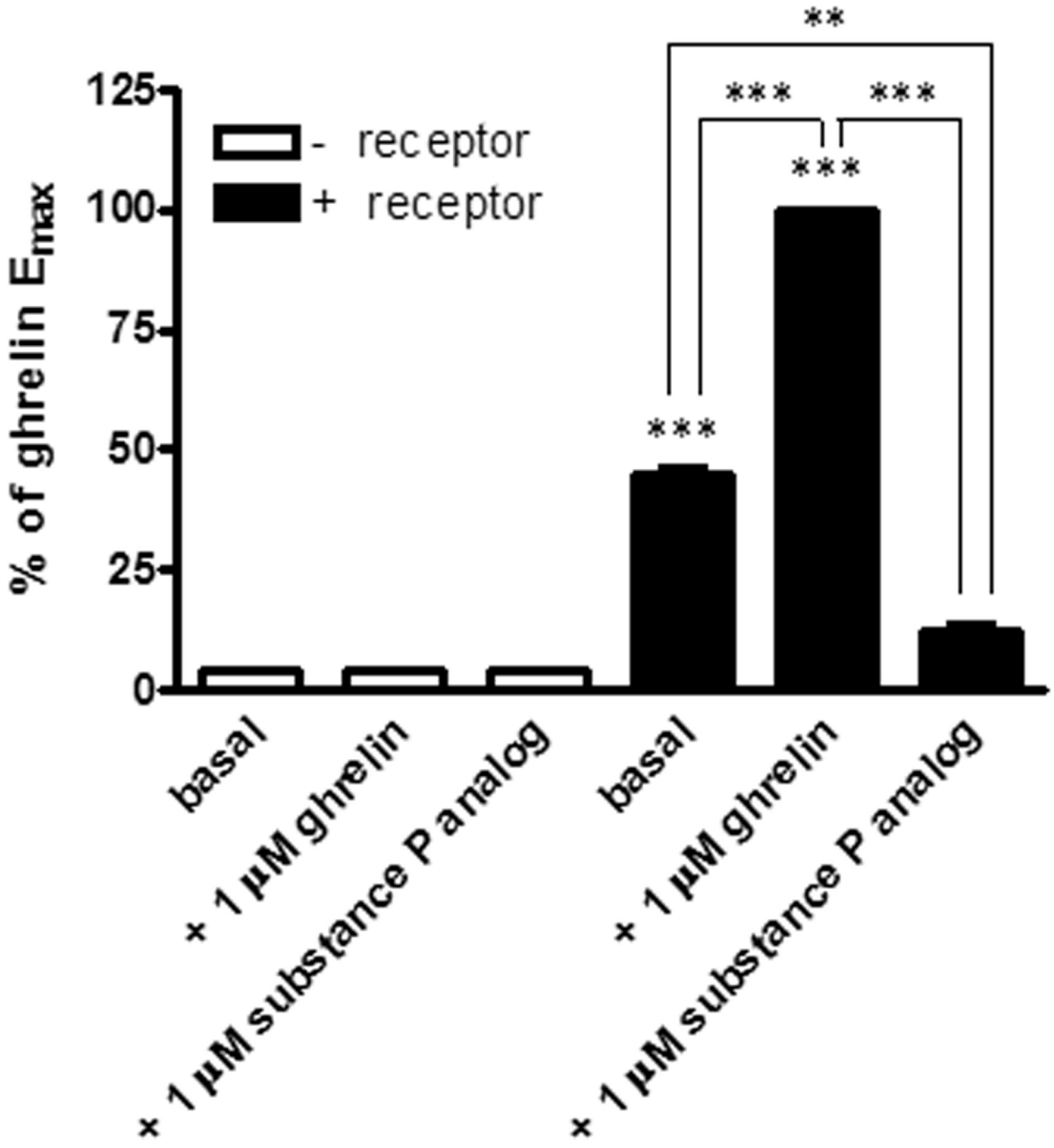

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Figure 2

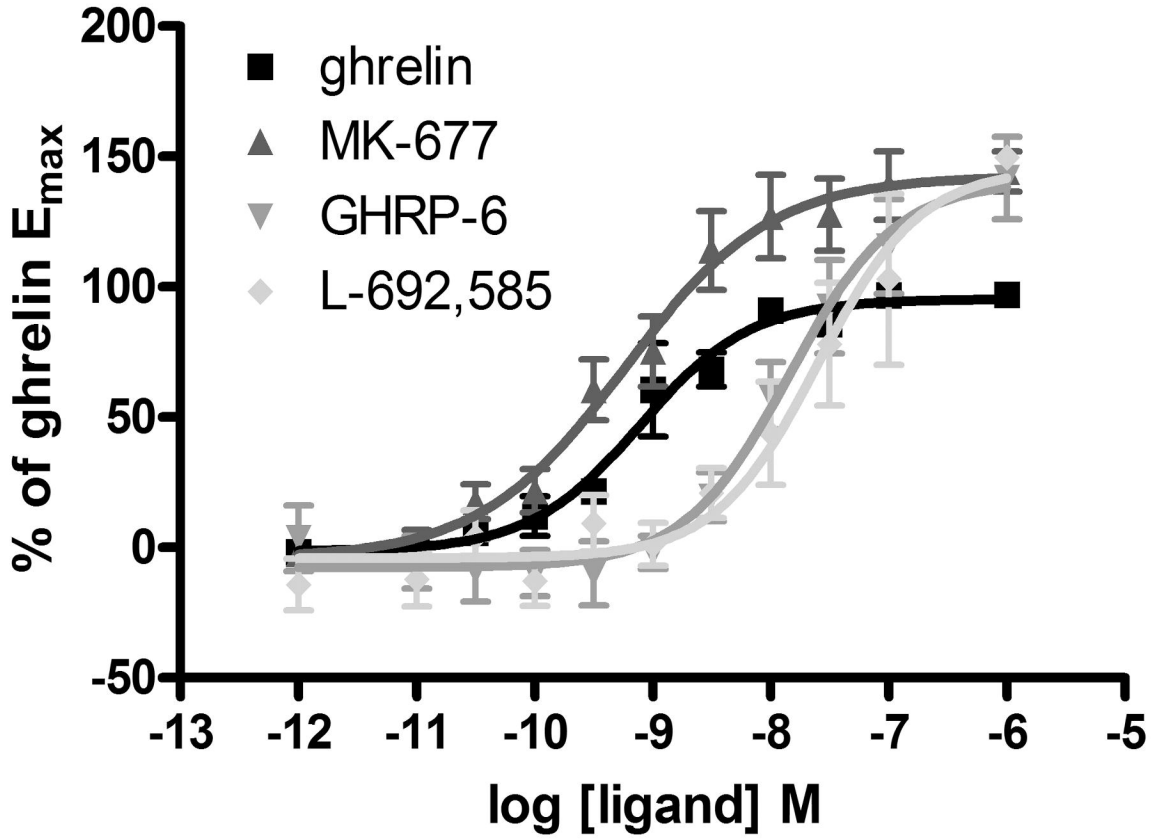

Figure 3

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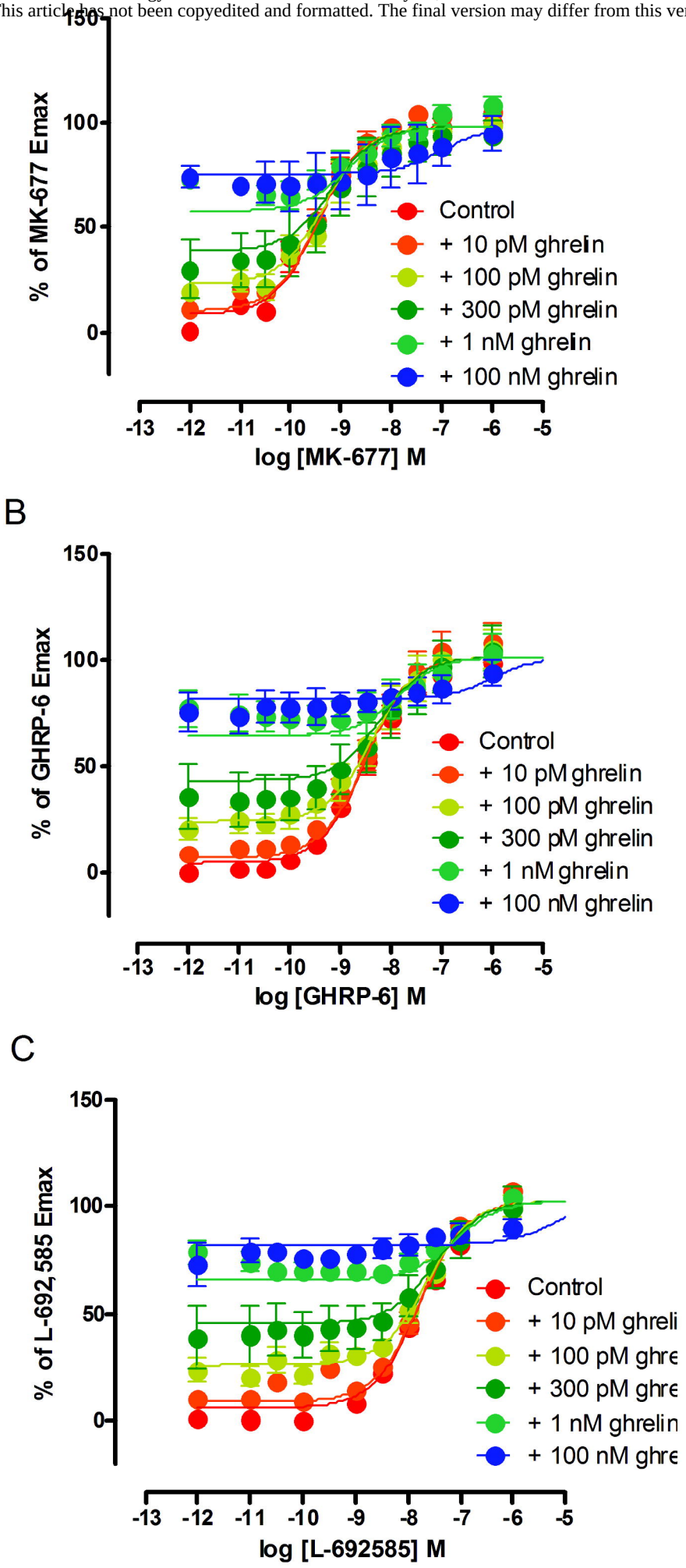

Figure 4

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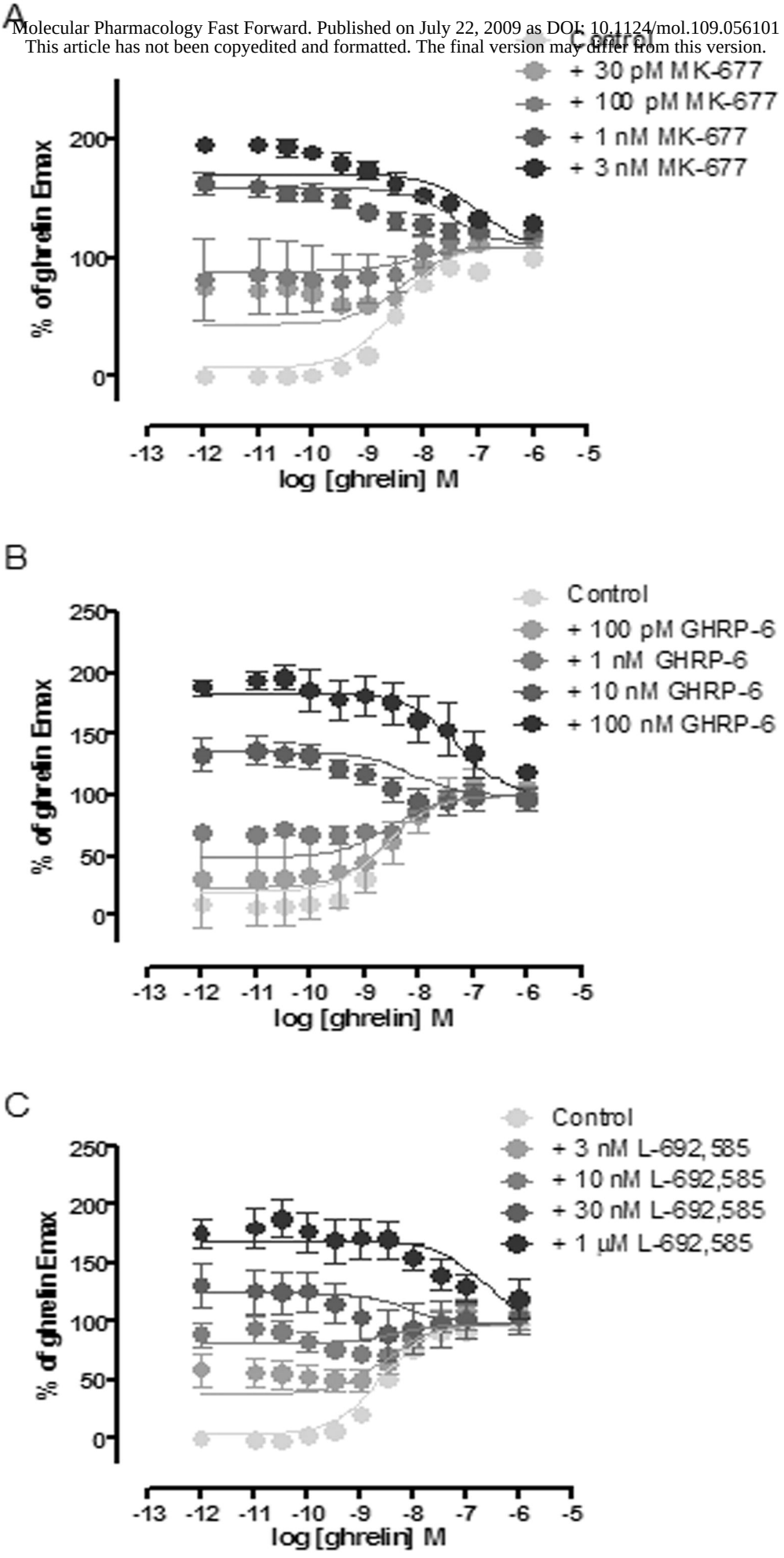

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Figure 5

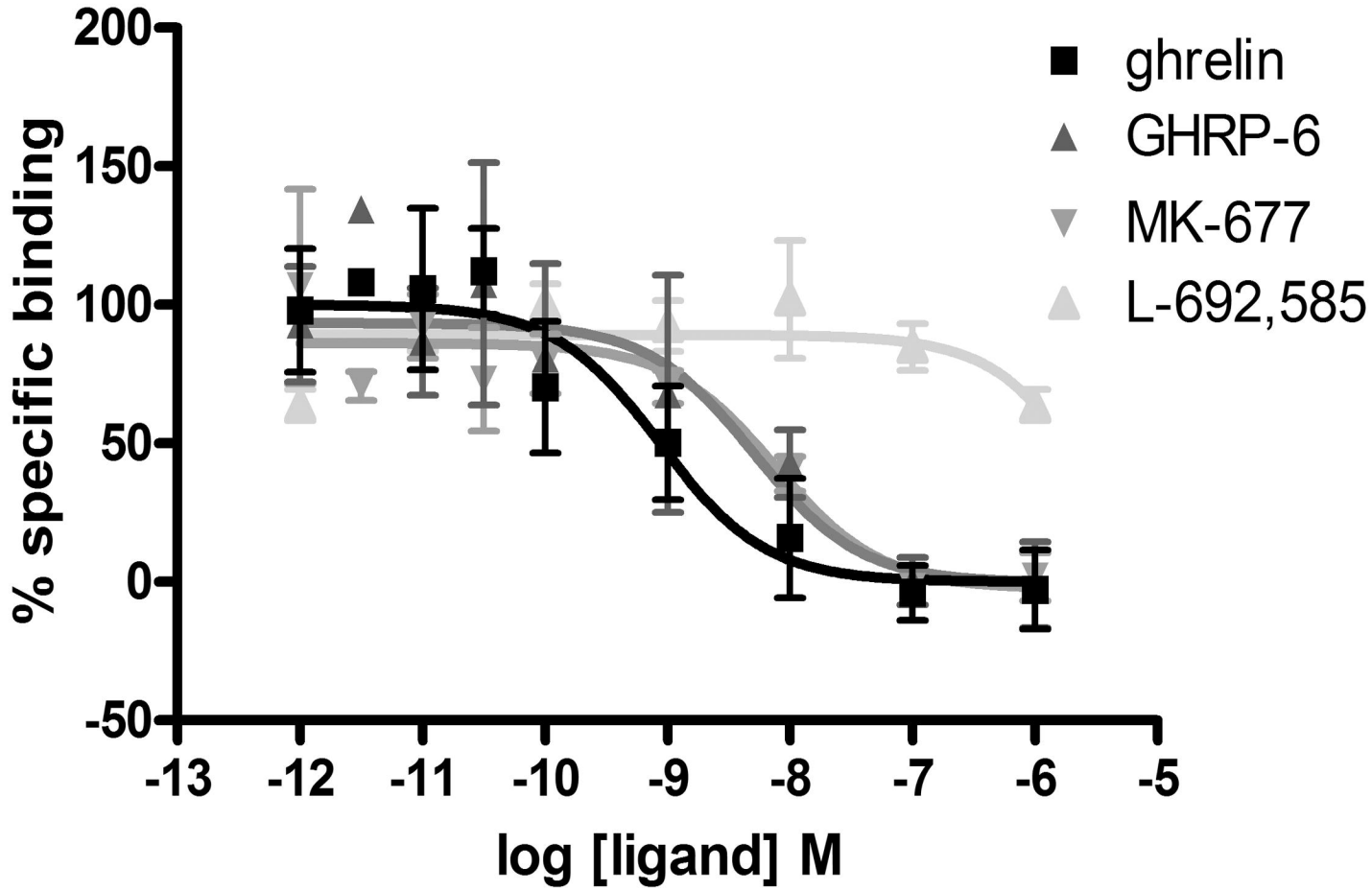

Figure 6

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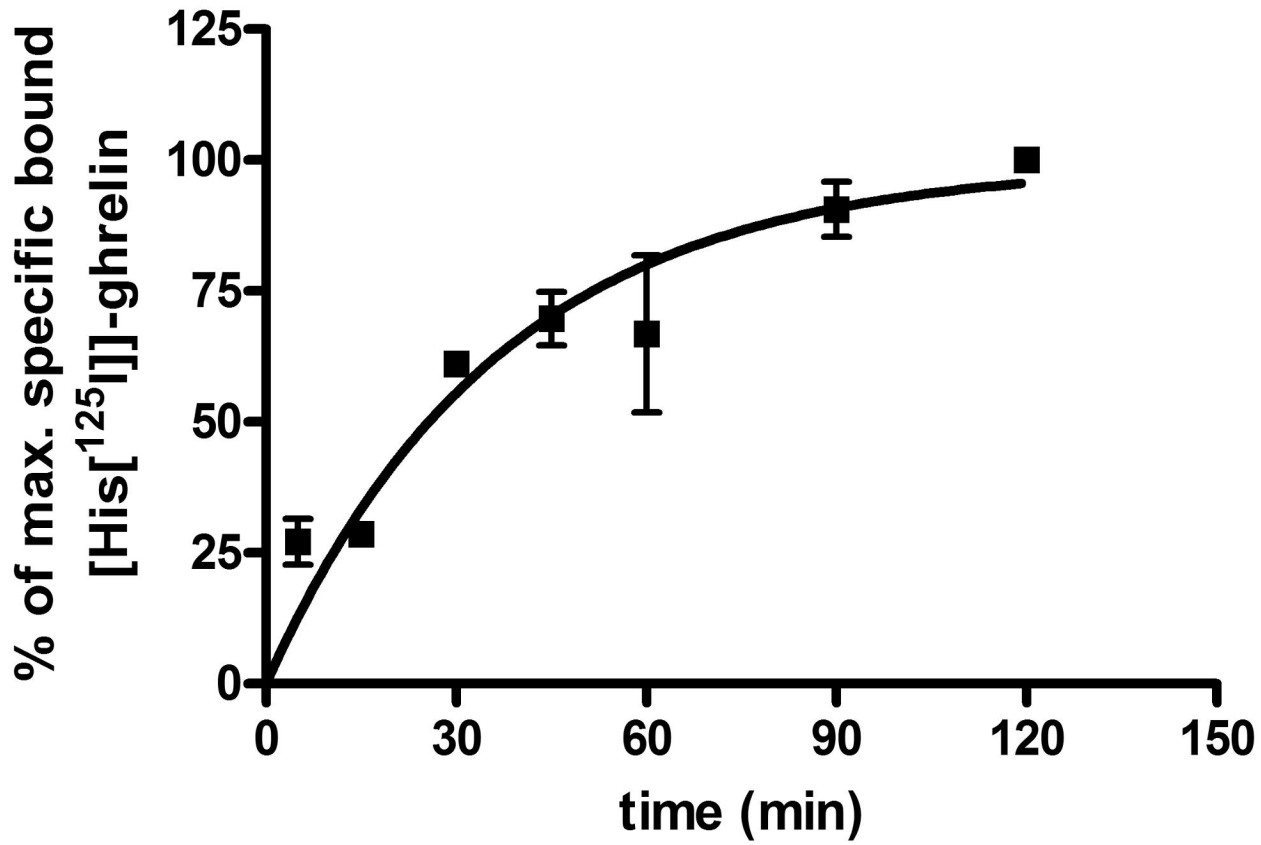
A



B

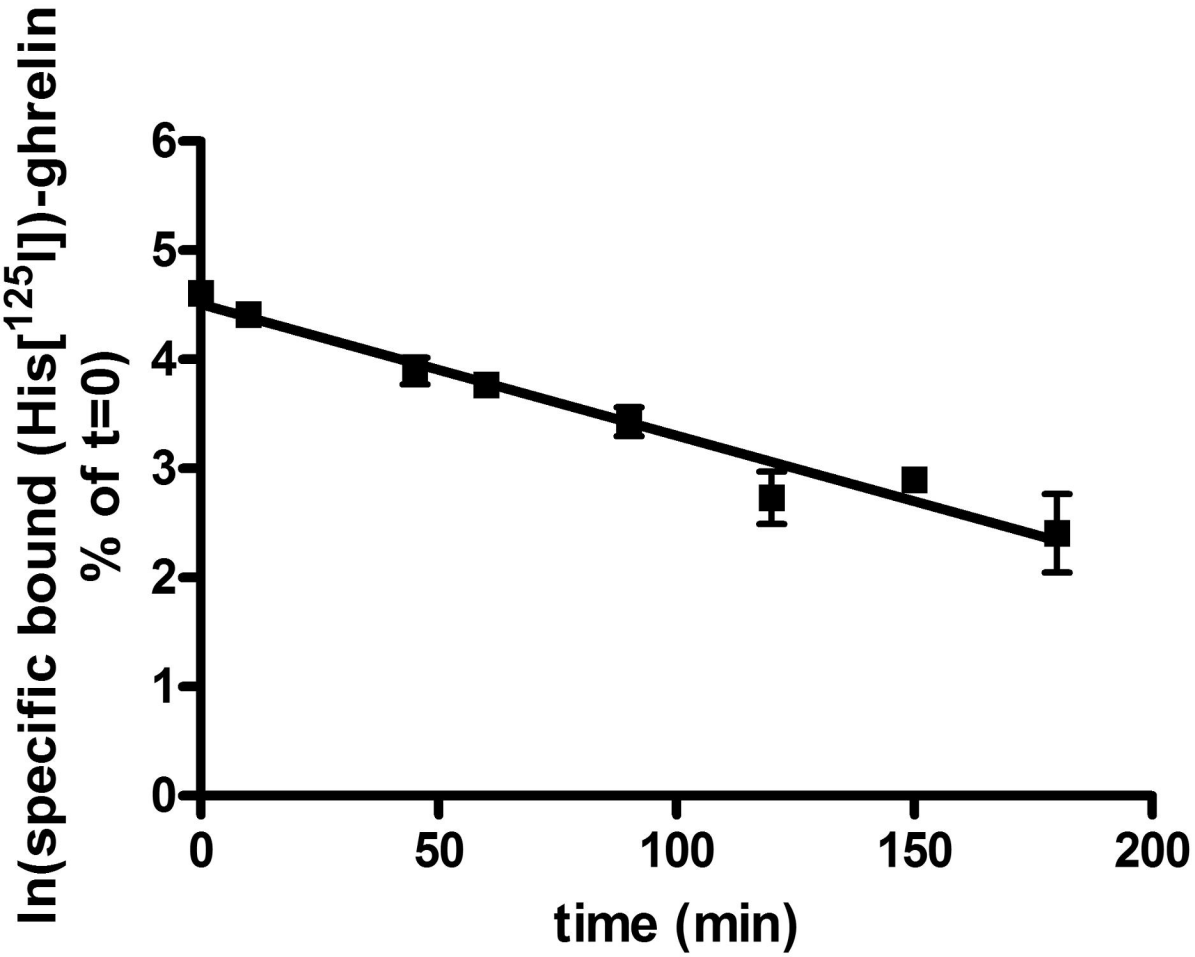


Figure 7

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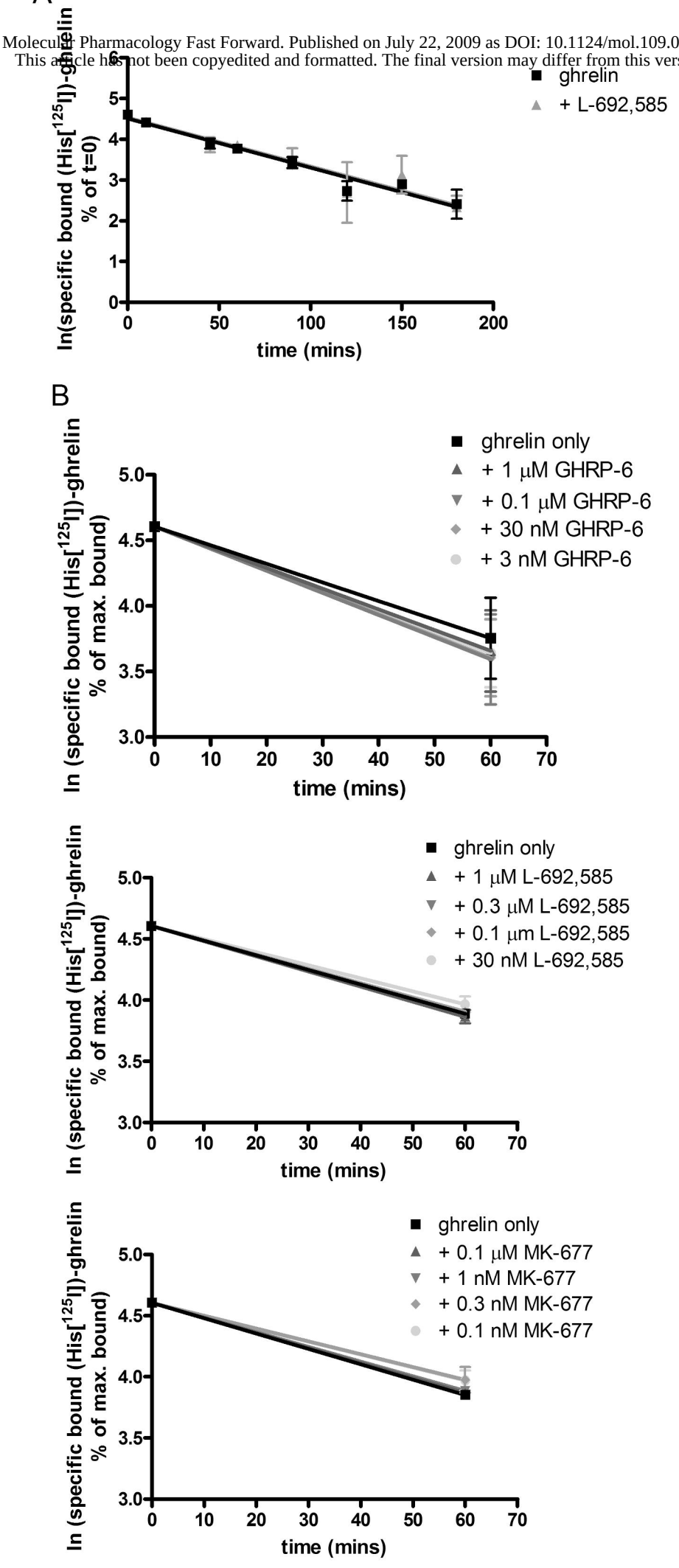

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Figure 8