Identification of new gonadotrophin-releasing hormone partial agonists

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Abstract

GnRH agonists or antagonists are currently utilized as therapeutic agents in a number of diseases. A side-effect of prolonged treatment with GnRH analogues is hypoestrogenism. In this study, we tested the *in vitro* potency of different GnRH analogues originally found to be partial agonists (i.e. analogues with decreased efficacy for activating or stimulating their cognate receptor) as well as novel analogues, to identify compounds that might potentially be useful for partial blockade of gonadotrophin release. Cultured COS-7 cells transiently expressing the rat or human GnRH receptor (GnRHR) were exposed to increasing concentrations (10^{-8} to 10^{-5} M) of GnRH analogues (c(4–10)[Asp⁴,DNaI⁶,Dpr¹⁰]-GnRH; c(4–10)[Dpr⁴,DNaI⁶,Asp¹⁰]-GnRH; c(4–10)[Cys^{4,10},DNaI⁶]-GnRH; c[Eaca¹,DNaI⁶]-GnRH; c[Gly¹,DNaI⁶]-GnRH; c[βAla¹,DTrp⁶]-GnRH; c[Dava¹,DNaI⁶]-GnRH; c[Gaba¹,DNaI⁶]-GnRH), and the ability of these analogues to provoke or antagonize GnRH-stimulated inositol phosphate production was assessed.

With both human and rat GnRHRs, c[Eaca¹,DNaI⁶]-GnRH, c[Gly¹,DNaI⁶]-GnRH, c[βAla¹,DTrp⁶]-GnRH and

c[Dava¹,DNaI⁶]-GnRH exhibited partial agonist activity (35–87% of the maximal efficacy shown by 10^{-6} M GnRH), whereas c[Gaba¹,DNaI⁶]-GnRH behaved as a partial agonist with the human GnRHR and as full agonist with the rat GnRHR. c(4–10)[Asp⁴,DNaI⁶,Dpr¹⁰]-GnRH and c(4–10)[Dpr⁴,DNaI⁶,Asp¹⁰]-GnRH exhibited full antagonist activity with both GnRHRs, and c(4–10)[Cys^{4,10},DNaI⁶]-GnRH was a weak, partial agonist with the human GnRHR and a full antagonist with the rat GnRHR. With the exception of c[Gaba¹,DNaI⁶]-GnRH stimulation of the human GnRHR, and c[Dava¹,DNaI⁶]-GnRH and c[Gaba¹,DNaI⁶]-GnRH stimulation of the rat GnRHR, all partial agonists also exhibited antagonist activity in the presence of the exogenous full agonist.

The results demonstrate that structurally similar analogues display variable potencies and efficacies *in vitro* for a specific GnRHR as well as for the human versus the rat GnRHR. Their ultimate *in vivo* usefulness to treat clinical conditions in which complete suppression of gonadotroph activity is not required remains to be investigated.

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Introduction

Gonadotrophin-releasing hormone (GnRH) is a key regulator of reproductive functions. This decapeptide is produced and secreted by specialized neurons predominantly located in the arcuate nucleus of the mediobasal hypothalamus and in the pre-optic area of the anterior hypothalamus (Conn & Crowley 1991, Ulloa-Aguirre & Timossi 2000). GnRH specifically interacts with its membrane receptor (R) in the gonadotrophs leading to both synthesis and secretion of the gonadotrophins luteinizing

hormone (LH) and follicle-stimulation hormone (FSH) (Conn *et al.* 1995, Millar *et al.* 2004). The release of GnRH from hypothalamic neurons occurs in a pulsatile manner, a feature that allows physiologic secretion of gonadotrophins (Santen & Bardin 1973, Knobil 1974). In fact, intermittent exposure of the GnRHR to the releasing hormone is important from a functional point of view, since it prevents desensitization (refractoriness) of the gonadotroph to a subsequent stimulus and allows for occurrence of the distinct rates and patterns of synthesis and release of LH and FSH that follow GnRH exposure (Belchetz *et al.* 1978).

The ability to suppress reproductive function by the continuous stimulation of the GnRHR by agonists (desensitization), or through continuous occupancy of the GnRHR with antagonists, has led to the development of GnRH agonists and antagonists currently employed as key therapeutic agents in a number of diseases, including cancer (Conn & Crowley 1991, Filicori 1994, Emons *et al.* 1997, Kiesel *et al.* 2002, Chabbert-Buffet *et al.* 2003). Analogues that capitalize on both mechanisms have been highly effective in suppressing the serum levels of gonadotrophins and steroid hormone production, provoking a chemical castration (Cusan *et al.* 1979, Conn & Crowley 1991, Filicori 1994, Emons *et al.* 1997, Ulloa-Aguirre & Timossi 2000, Kiesel *et al.* 2002, Chabbert-Buffet *et al.* 2003). Nevertheless, complete and prolonged suppression of gonadotrophin secretion and gonadal steroids by GnRH analogues frequently results in symptoms of oestrogen deficiency (DeFazio *et al.* 1983, Matta *et al.* 1987, Johansen *et al.* 1988, Tummon *et al.* 1988, Conn & Crowley 1991, Fogelman 1992, Pierce *et al.* 2000). Furthermore, the use of lower doses of GnRH-agonist analogues to allow for some residual pituitary-gonadal function has failed to fully overcome this adverse effect (Conn & Crowley 1991). In this scenario, partial GnRH agonists (i.e. agonists that exhibit decreased efficacy for activating or stimulating their cognate receptor thereby eliciting lower maximal responses than a full agonist) may potentially be useful in those conditions in which complete suppression of gonadal steroid secretion is not required, particularly when long-term treatment is needed and hormonal replacement add-back therapy is contraindicated or proven to be inefficient (Pierce *et al.* 2000).

Among the thousands of GnRH analogues described in the literature, very few exhibited partial agonism and were forgotten once better antagonists became available. The first observation of a GnRH partial agonist ([Gly²]-GnRH) led to the design of the first GnRH antagonist, des-His²-GnRH (Vale *et al.* 1972). Interestingly, substitutions other than Gly at position 2 of GnRH (such as L-amino acids) led to low potency full agonists whereas substitutions by a D-amino acid led to full antagonists (Karten & Rivier 1986). It is only with the synthesis of cyclic GnRH analogues that a new family of GnRH partial agonists was discovered. This family encompasses sequences that are N- to C-terminally cyclized (Rivier *et al.* 1986). In view of the fact that cyclization could bring partial agonism, we synthesized a second family of analogues characterized by sequences that are cyclized through the side chains of amino acids at positions 4 and 10. Three members of the first family were described in a preliminary report. c[Eaca¹,DNaI⁶]-GnRH, c[βAla¹,DNaI⁶]-GnRH and c[Gaba¹,DNaI⁶]-GnRH are partial agonists with high binding affinity (Rivier *et al.* 1986). Members of the second family are disclosed here for the first time and were tested for their *in vitro* properties.

Materials and Methods

Materials

Synthetic GnRH was provided by the NIDDK National Hormone and Peptide Program through Dr A F Parlow (Torrance, CA, USA). The GnRH agonist, Buserelin ([D-tert-butyl-Ser⁶,des-Gly¹⁰,Pro⁹-ethylamide]-GnRH) was a kind gift of Hoechst-Roussel Pharmaceutical (Somerville, NJ, USA). The following peptides were synthesized at the Salk Institute (La Jolla, CA, USA): c(4-10)[Cys^{4,10},DNaI⁶]-GnRH, c[βAla¹,DTrp⁶]-GnRH, c[Dava¹,DNaI⁶]-GnRH, c[Gly¹,DNaI⁶]-GnRH, c[Eaca¹,DNaI⁶]-GnRH, c[Gaba¹,DNaI⁶]-GnRH, c(4-10)[Dpr⁴,DNaI⁶,Asp¹⁰]-GnRH and c(4-10)[Asp⁴,DNaI⁶,Dpr¹⁰]-GnRH. Peptides were diluted in PBS/10% dimethylsulphoxide (DMSO) at a 1 µg/µl concentration and the stocks were stored frozen at -70 °C until they were used. The expression vector pcDNA3.1, Dulbecco's modified Eagle's medium (DMEM), OPTI-MEM, lipofectamine and PCR reagents were purchased from Invitrogen. Restriction enzymes, modified enzymes and competent cells for subcloning were purchased from Promega. Other reagents were of the highest degree of purity available from commercial sources.

Vectors

Wild-type (WT) rat (r) GnRHR cDNA in pcDNA1 was subcloned into pcDNA3.1 at BamHI and XhoI restriction enzyme sites. The WT human (h) GnRHR cDNA in pcDNA3 was also subcloned into pcDNA3.1 at KpnI and XbaI restriction enzyme sites.

Transient transfection of COS-7 cells

WT rGnRH and hGnRH receptors were transiently expressed in COS-7 cells. COS-7 cells were maintained at 37 °C in growth medium (DMEM) containing 10% fetal calf serum (FCS) (Life Technologies) and 20 µg/ml gentamicin (Gemini Bioproducts, Calabasas, CA, USA) in a humidified 5% CO₂ atmosphere. The cells (10⁵ cells/well) were seeded in 24-well plates (Costar, Cambridge, MA, USA). Twenty-four hours after plating, the cells were transfected with 0.075 µg (for inositol phosphate (IP) production) or 0.1 µg (for ¹²⁵I-labelled buserelin binding) of cDNA/well using 2 µl lipofectamine in 0.25 ml OPTI-MEM. Five hours later, 0.25 ml DMEM containing 20% FCS was added to each well. Twenty-four hours after the start of transfection the medium was replaced with fresh growth medium and the cells were allowed to grow for another 24 h before the functional assay (IP production).

Measurement of IP production

Forty-eight hours after the start of transfection, COS-7 cells transfected with the WT rat or human GnRH

Figure 1 Stimulation of IP production in transiently transfected COS-7 cells with the WT hGnRHR (A) or WT rGnRHR (B) by different GnRH peptide analogues. IP production was determined after a 2-h incubation in the presence of increasing concentrations of each peptide as described in Materials and Methods. Data are the means $\pm$ S.E.M. of a representative experiment performed in triplicate and repeated three or more times.

receptor cDNAs were washed twice with DMEM/0.1% BSA and intracellular inositol lipids were labelled with 4 μ Ci/ml [3 H]myo-inositol in DMEM (inositol free) for 18 h at 37 °C. After the preloading period, the cells were washed twice in DMEM (inositol free) containing 5 mM LiCl and incubated for 2 h at 37 °C in the absence or presence of GnRH (10^{-10} , 10^{-8} and 10^{-6} M), buserelin (10^{-11} , 10^{-9} and 10^{-7} M) or increasing concentrations (10^{-11} to 10^{-5} M) of the different GnRH analogues in DMEM (inositol free)/LiCl prepared by serial dilutions of the original stocks in DMEM. Alternatively, transfected cells were co-incubated with 10^{-8} M GnRH and 10^{-8} to 10^{-5} M concentrations of the GnRH analogues. At the end of the incubation period, the medium was removed and 1 ml of 0.1 M formic acid was added to each well. The cells were frozen at -20 °C until assayed for IP production. IP accumulation was determined by Dowex anion-exchange chromatography and liquid scintillation spectroscopy as previously described (Huckle & Conn 1987).

Receptor binding assay

COS-7 cells were transiently transfected with the WT rat or human GnRH receptor cDNAs as described above. Twenty-seven hours after the start of transfection, the cells were washed twice with warm DMEM/0.1% BSA/10 mM HEPES buffer and cultured in DMEM for 18 h before the addition of 125 I-buserelin (specific activity, 700 μ Ci/ μ g; $\sim 10^6$ c.p.m./0.5 ml; pH 7.4). Cells were incubated at room temperature for 90 min in the presence or absence of excess (2 μ M) GnRH plus 125 I-buserelin or 125 I-buserelin plus increasing concentrations (10^{-10} to 10^{-5} M) of GnRH or the different GnRH analogues.

Thereafter, the medium was removed, the plates containing the cells were placed on ice, washed twice with ice-cold PBS; the cells were then solubilized by the addition of 0.2 M NaOH/0.1% SDS. Aliquots of samples were then transferred to glass tubes and counted in a gamma counter (Packard Instruments, Downers Grove, IL, USA). Specific binding was calculated by subtracting non-specific binding (binding measured in the presence of 2 μ M GnRH) from total binding (no GnRH added).

Statistical analysis

Data shown are the means $\pm$ S.E.M. from three or more experiments in triplicate incubations. In all experiments, the S.D. was typically less than 10% of the corresponding mean. Within-group comparisons were made by one-way ANOVA followed by Tukey's HSD test; between-group comparisons were made using Student's unpaired *t* test. Values of $P < 0.05$ were considered statistically significant. Half-maximal doses (ED_{50}) and concentrations giving 50% of maximal inhibition (IC_{50}) were calculated using the software Origin 7.0 (OriginLab Co, Northampton, MA, USA) and fitted to a sigmoidal dose-response curve.

Results

Stimulation of IP turnover by GnRH and GnRH analogues

Synthetic GnRH, and its metabolically stable superagonist buserelin, provoked concentration-dependent increments in IP production by COS-7 cells transiently expressing either the WT rGnRHR or the WT hGnRHR (Fig. 1).

Table 1 ED₅₀ and IC₅₀ values of different GnRH peptide analogues in COS-7 cells transiently expressing the WT rGnRHR or WT hGnRHR

Peptide	ED ₅₀ for agonist effect (nM)		IC ₅₀ on 10 ⁻⁸ GnRH (nM)	
	hGnRHR	rGnRHR	hGnRHR	rGnRHR
Buserelin	0.04 ± 0.01 ^a	0.05 ± 0.01 ^{a,f}	—	—
GnRH	0.42 ± 0.06 ^{b,c,g*}	1.5 ± 0.48 ^b	—	—
c(4–10)[Asp ⁴ ,DNaI ⁶ ,Dpr ¹⁰]-GnRH	—	—	57 ± 9 ^{a*}	14 ± 3 ^a
c(4–10)[Dpr ⁴ ,DNaI ⁶ ,Asp ¹⁰]-GnRH	—	—	147 ± 27 ^{b*}	18 ± 3 ^a
c(4–10)[Cys ^{4,10} ,DNaI ⁶]-GnRH	941 ± 194 ^e	3860 ± 1159 ^c	308 ± 35 ^{c*}	85 ± 19 ^b
c[Eaca ¹ ,DNaI ⁶]-GnRH	0.17 ± 0.04 ^{d*}	0.02 ± 0.01 ^a	—	—
c[Gly ¹ ,DNaI ⁶]-GnRH	0.19 ± 0.02 ^{c,d}	0.11 ± 0.02 ^d	—	—
c[βAla ¹ ,DTrp ⁶]-GnRH	1.1 ± 0.40 ^{d,f*}	13.1 ± 1.80 ^e	—	—
c[Dava ¹ ,DNaI ⁶]-GnRH	0.48 ± 0.13 ^{c,d,f}	0.39 ± 0.10 ^d	—	—
c[Gaba ¹ ,DNaI ⁶]-GnRH	0.34 ± 0.08 ^{f,g*}	0.05 ± 0.01 ^f	—	—

Data are means ± S.E.M. of three independent experiments in triplicate incubations. Means identified by different superscript letters in the same column are significantly (*P*<0.05) different. **P*<0.05 vs rat GnRHR system. Gaba, γ-aminobutyric acid; Eaca, ε-aminocaproic acid; Dava, δ-aminovaleric acid; DNaI=β-(2-naphthyl)-D-alanine; Dpr, 2,3-diaminopropionic acid.

Table 1 shows the ED₅₀ values of GnRH, buserelin and the GnRH peptides tested. In the human GnRHR system, c[Eaca¹,DNaI⁶]-GnRH and c[Gly¹,DNaI⁶]-GnRH presented slightly lower ED₅₀ values than GnRH but considerably reduced fractions of maximal response (Table 2). The ED₅₀ values of c[Dava¹,DNaI⁶]-GnRH and c[Gaba¹,DNaI⁶]-GnRH were similar to that shown by GnRH. Compared with GnRH, c[Eaca¹,DNaI⁶]-GnRH, c[Gly¹,DNaI⁶]-GnRH, c[Dava¹,DNaI⁶]-GnRH and c[Gaba¹,DNaI⁶]-GnRH exhibited lower ED₅₀ values than GnRH with the rGnRHR (Table 1); nevertheless, the fraction of maximal response of all of these GnRH peptides (except c[Gaba¹,DNaI⁶]-GnRH) was significantly attenuated. Table 2 shows the efficacy to provoke maximal IP production and to inhibit GnRH-stimulated IP production of the different GnRH peptides. According to the responses observed in the particular receptor systems used, the operational definitions of agonist, partial agonist or antagonist were employed to define the type of activity of each analogue.

In the hGnRHR system, c(4–10)[Cys^{4,10},DNaI⁶]-GnRH and c[Eaca¹,DNaI⁶]-GnRH behaved as weak, partial agonists (13–35% of the maximal response observed with GnRH or buserelin (Fig. 1A and Table 2)); c[Gly¹,DNaI⁶]-GnRH, c[βAla¹,DTrp⁶]-GnRH, c[Dava¹,DNaI⁶]-GnRH and c[Gaba¹,DNaI⁶]-GnRH were more efficacious partial agonists (73–87% efficacy). c(4–10)[Asp⁴,DNaI⁶,Dpr¹⁰]-GnRH and c(4–10)[Dpr⁴,DNaI⁶,Asp¹⁰]-GnRH had no effect on IP production in this receptor system (Tables 1 and 2). In the rGnRHR system, c[Eaca¹,DNaI⁶]-GnRH, c[Gly¹,DNaI⁶]-GnRH, c[βAla¹,DTrp⁶]-GnRH, and c[Dava¹,DNaI⁶]-GnRH behaved as partial agonists on GnRHR activation (44–87% of the maximal efficacy shown by either 10⁻⁶ M GnRH or 10⁻⁷ M buserelin); c[Gaba¹,DNaI⁶]-GnRH exhibited the highest efficacy (98% of the efficacy shown by GnRH or buserelin) in this particular GnRHR system and thus

behaved as a full agonist (Table 2). No agonist activity was detected for the c(4–10)[Asp⁴,DNaI⁶,Dpr¹⁰]-GnRH, c(4–10)[Dpr⁴,DNaI⁶,Asp¹⁰]-GnRH and c(4–10)[Cys^{4,10},DNaI⁶]-GnRH compounds (Fig. 1B).

Inhibition of GnRH-stimulated IP accumulation by GnRH analogues

In both GnRHR systems, c(4–10)[Asp⁴,DNaI⁶,Dpr¹⁰]-GnRH and c(4–10)[Dpr⁴,DNaI⁶,Asp¹⁰]-GnRH behaved as full antagonists of GnRH (Table 2). c(4–10)[Cys^{4,10},DNaI⁶]-GnRH behaved as a strong antagonist with the rGnRHR, but not in the hGnRHR system (Fig. 2A and B, and Tables 1 and 2). In the rGnRHR system, c[Eaca¹,DNaI⁶]-GnRH and c[Gly¹,DNaI⁶]-GnRH inhibited (by 30–40%) the 10⁻⁸ M GnRH-mediated IP response, whereas in the hGnRHR system, only the c(4–10)[Cys^{4,10},DNaI⁶]-GnRH and c[Eaca¹,DNaI⁶]-GnRH analogues showed a similar effect. In contrast to the effects observed with the rGnRHR, in the human receptor system, c[Gly¹,DNaI⁶]-GnRH marginally (15%) inhibited GnRH stimulation of IP accumulation. In both GnRHRs, c[βAla¹,DTrp⁶]-GnRH, c[Dava¹,DNaI⁶]-GnRH and c[Gaba¹,DNaI⁶]-GnRH showed substantially decreased or null efficacy to inhibit GnRH-stimulated IP production.

Effect of GnRH analogues on ¹²⁵I-buserelin binding

All GnRH peptides inhibited specific ¹²⁵I-buserelin binding in a dose-related manner (Fig. 3). Relative receptor levels were higher for the rGnRHR system (62.0 ± 4.7 pmol/10⁵ cells) than in the hGnRHR system (1.2 ± 0.22 pmol/10⁵ cells). The potency of the peptides to inhibit binding of radiolabelled buserelin (Table 3) did not absolutely correlate with IP production in response to buserelin (compare Figs 1 and 3). In fact,

Table 2 Maximal stimulation of IP production and inhibition of GnRH-stimulated IP production by different GnRH peptides in COS-7 cells transiently expressing the WT rGnRHR or WT hGnRHR

Peptide	Agonist effect (% maximal response)		Inhibitory effect on 10^{-8} M GnRH (% maximal inhibition)		Analogue type	
	hGnRHR	rGnRHR	hGnRHR	rGnRHR	hGnRHR	rGnRHR
	100	100	—	—	Agonist	Agonist
GnRH or buserelin	100	100	100	100	Antagonist	Antagonist
c(4–10)[Asp ⁴ ,DNal ⁶ ,Dpr ¹⁰]-GnRH	0.2 ± 0.2 ^a	0.1 ± 0.1 ^a	99.9 ± 0.05 ^a	100	Antagonist	Antagonist
c(4–10)[Dpr ⁴ ,DNal ⁶ ,Asp ¹⁰]-GnRH	0.3 ± 0.1 ^a	0.1 ± 0.1 ^a	99.9 ± 0.05 ^a	100	Antagonist	Antagonist
c(4–10)[Cys ^{4,10} ,DNal ⁶]-GnRH	13.0 ± 1.0 ^{b*}	6.2 ± 0.6 ^a	82.01 ± 0.33 ^{b*}	90.8 ± 0.20 ^a	Weak, partial agonist	Antagonist
c[Eaca ¹ ,DNal ⁶]-GnRH	35.0 ± 1.0 ^{c*}	45.0 ± 1.0 ^b	69.4 ± 0.75 ^{c*}	32.9 ± 2.7 ^b	Weak, partial agonist	Weak, partial agonist
c[Gly ¹ ,DNal ⁶]-GnRH	73.0 ± 1.0 ^{d*}	44.0 ± 2.0 ^b	15.4 ± 0.87 ^{d*}	39.2 ± 1.0 ^b	Partial agonist	Weak, partial agonist
c[βAla ¹ ,DTrp ⁶]-GnRH	77.0 ± 1.0 ^{d*}	58.0 ± 1.0 ^b	13.1 ± 1.81 ^{d*}	7.7 ± 0.89 ^c	Partial agonist	Weak, partial agonist
c[Dava ¹ ,DNal ⁶]-GnRH	79.0 ± 3.0 ^d	87.0 ± 6.0 ^c	21.7 ± 2.59 ^f	0	Partial agonist	Partial agonist
c[Gaba ¹ ,DNal ⁶]-GnRH	87.0 ± 2.0 ^e	98.0 ± 6.0 ^c	0	0	Partial agonist	Agonist

Data are means ± SEM, of three independent experiments in triplicate incubations. Data are expressed as the fraction of maximally stimulated IP response to GnRH analogues (at a 10^{-5} M concentration) relative to 10^{-6} M GnRH (and 10^{-7} M Buserelin) or the percentage of maximal inhibition on the 10^{-8} M GnRH-stimulated IP response. Means identified by different superscript letters in the same column are significantly ($P < 0.05$) different. * $P < 0.05$ vs rat GnRHR system. For abbreviations see Table 1.

the full antagonists c(4–10)[Asp⁴,DNal⁶,Dpr¹⁰]-GnRH and c(4–10)[Dpr⁴,DNal⁶,Asp¹⁰]-GnRH were the most potent analogues for inhibiting ¹²⁵I-buserelin binding with both GnRHR systems (Table 3).

Discussion

The effects of GnRH are mediated via LH and FSH released in response to the releasing decapeptide and via sex steroid hormones secreted by the gonads in response to gonadotrophin stimulation. Full agonists used as desensitizing agents render the target cell refractory, whereas antagonists block the binding site of the GnRHR to endogenous occupancy without coupling to the receptor or triggering signal transduction (Cusan *et al.* 1979, Conn & Crowley 1991, Ortmann *et al.* 2002). Either approach generally results in oestrogen deficiency, thereby limiting their usefulness as therapeutic agents when partial desensitization is desired.

In contrast to full antagonists, which bind equally to both the inactive and active receptor conformations, partial agonists are characterized by exhibiting higher affinity for the active conformation than for its inactive conformation, but with less selectivity towards the active conformation than the strong agonists (Brink *et al.* 2004). At high concentrations, partial agonists elicit a lower maximal response in a particular biological system than a defined standard and may act either as a functional agonist or a functional antagonist, depending on the levels of the naturally occurring agonist (Lipworth & Grove 1997, Pliska 1999, Lieberman 2004). Because of their particular selectivity towards different receptor states and low potential to provoke side-effects, these compounds currently represent attractive options in the treatment of a variety of diseases (Callahan 2002, Frolund *et al.* 2002, Doggrel 2004, Gao & Jacobson 2004, Lieberman 2004, Reynolds 2004, Bolonna & Kerwin 2005).

In the present study, we analyzed the *in vitro* effects of different GnRH peptide analogues on intracellular signalling triggered by the activation of two structurally similar GnRH receptors. Using the rGnRHR system, we identified three GnRH-antagonist analogues (c(4–10)[Asp⁴,DNal⁶,Dpr¹⁰]-GnRH, c(4–10)[Dpr⁴,DNal⁶,Asp¹⁰]-GnRH and c(4–10)[Cys^{4,10},DNal⁶]-GnRH) and four compounds that behaved as partial agonists (c[Eaca¹,DNal⁶]-GnRH, c[Gly¹,DNal⁶]-GnRH, c[βAla¹,DTrp⁶]-GnRH and c[Dava¹,DNal⁶]-GnRH); these compounds, and c[Gaba¹,DNal⁶]-GnRH (a full agonist at the highest concentration tested in the rGnRHR system), behave as partial agonists with the hGnRHR. With the exception of c[Gaba¹,DNal⁶]-GnRH, and to a lesser extent c[Gly¹,DNal⁶]-GnRH, c[βAla¹,DTrp⁶]-GnRH and c[Dava¹,DNal⁶]-GnRH with the hGnRHR, and c[βAla¹,DTrp⁶]-GnRH, c[Dava¹,DNal⁶]-GnRH and c[Gaba¹,DNal⁶]-GnRH in the rGnRHR system, partial GnRH agonists also exhibited

Figure 2 Inhibition of GnRH-stimulated IP production by different peptides. The peptides (10^{-8} to 10^{-5} M) were preincubated with 10^{-8} M GnRH before stimulating COS-7 cells transiently transfected with the WT hGnRHR (A) or WT rGnRHR (B). IP production was determined after a 2-h incubation. Data are means $\pm$ S.E.M. of a representative experiment performed in triplicate and repeated three or more times.

significant antagonist activity in the presence of the exogenous full agonist, which is an intrinsic pharmacologic feature of this class of compounds (Lipworth & Grove 1997, Pliska 1999).

It was interesting to find that some of the analogues studied displayed different potencies and even differential effects when tested with the rat and human GnRHRs, which share 88% in amino acid identity (Millar *et al.* 2004). For example, c[Gly¹,DNaI⁶]-GnRH and c[Ala¹,DTrp⁶]-GnRH exhibited lower efficacies (fraction of maximal response, 0.4–0.6) to activate the rGnRHR than the human receptor (0.7–0.8 times the efficacy of GnRH). Furthermore, c(4–10)[Cys^{4,10},DNaI⁶]-GnRH behaved as a full antagonist in the rGnRHR system,

whereas in the human receptor it behaved as a weak, partial agonist. Although this variability of effects may be due to differences in relative receptor levels between the two GnRHR systems, it is also possible that positioning of the analogues in each GnRH receptor may potentially impede an optimal interaction of the analogue with key receptor domains involved in ligand binding and/or receptor activation, thereby altering its ability to stabilize the active state of the receptor. In this scenario, subtle structural differences between these receptors would allow the agonists to drive with different efficacies the equilibrium between the inactive and active conformations in favour of the latter. In the case of c(4–10)[Cys^{4,10},DNaI⁶]-GnRH, the weak partial agonist activity with the

Figure 3 Inhibition of ¹²⁵I-buserelin binding by GnRH and GnRH peptides. COS-7 cells transiently expressing the WT hGnRHR (A) or WT rGnRHR (B) were exposed to ¹²⁵I-buserelin for 90 min in the presence or absence of excess (2 µM) GnRH or increasing concentrations (10^{-10} to 10^{-5} M) of the different GnRH analogues. Data are means $\pm$ S.E.M. of a representative experiment performed in triplicate and repeated at least three times.

Table 3 IC₅₀ doses of GnRH and different GnRH peptide analogues to inhibit ¹²⁵-buserelin binding in COS-7 cells transiently expressing the WT rGnRHR or WT hGnRHR

Peptide	IC ₅₀ on ¹²⁵ -buserelin binding (nM)	
	hGnRHR	rGnRHR
GnRH	0.36 ± 0.07 ^{a*}	4.33 ± 0.32 ^a
c(4–10)[Asp ⁴ ,DNaI ⁶ ,Dpr ¹⁰]-GnRH	0.14 ± 0.01 ^{b*}	0.04 ± 0.01 ^b
c(4–10)[Dpr ⁴ ,DNaI ⁶ ,Asp ¹⁰]-GnRH	0.21 ± 0.03 ^{a,b*}	0.08 ± 0.01 ^c
c(4–10)[Cys ^{4,10} ,DNaI ⁶]-GnRH	22 ± 2.80 ^{c*}	9.2 ± 0.80 ^d
c[Eaca ¹ ,DNaI ⁶]-GnRH	7.4 ± 1.80 ^{d*}	3.6 ± 0.50 ^a
c[Gly ¹ ,DNaI ⁶]-GnRH	15 ± 0.50 ^{c*}	11.4 ± 1.0 ^{e,d}
c[βAla ¹ ,DTrp ⁶]-GnRH	953 ± 66 ^{e*}	249 ± 30 ^f
c[Dava ¹ ,DNaI ⁶]-GnRH	381 ± 72 ^{f*}	0.74 ± 0.11 ^g
c[Gaba ¹ ,DNaI ⁶]-GnRH	0.22 ± 0.01 ^{a*}	5.1 ± 0.80 ^a

Data are means ± S.E.M. of three independent experiments in triplicate incubations. Means identified by different superscript letters in the same column are significantly ($P < 0.05$) different. * $P < 0.05$ vs rat GnRHR system. For abbreviations see Table 1.

hGnRHR may arise from the interaction of the peptide with amino acid residues or domains that are unique to the human receptor (e.g. at the exoloop 2 or intraloop 2, which share 76 and 62% amino acid identity with the corresponding domains in the rGnRHR); in this setting, the particular structure of the rGnRHR antagonist might ultimately determine a preference for binding to the active receptor conformation of the hGnRHR and to both the inactive and active conformations of the rat counterpart, as would be expected for antagonist analogues. In fact, in a number of G protein-coupled receptors, including the GnRHR, mutations or species-dependent structural differences in either transmembrane domains (Strader *et al.* 1989, Claude *et al.* 1996, Noda *et al.* 1996, Groblewski *et al.* 1997, Han *et al.* 1997, Spivak *et al.* 1997), intraloops (Morin *et al.* 1998, Wurch *et al.* 1999) or exoloops (Sun *et al.* 2001) may be limiting, to a variable extent, the level of maximal stimulation. It should be emphasized, however, that the effects of the compounds tested may be cell-specific and that depending on the relative levels of receptor, G proteins and other downstream effectors present in a particular tissue *in vivo* (i.e. the pituitary gland), their net effects observed might vary.

We noticed that some of the compounds tested (e.g. c[Gly¹,DNaI⁶]-GnRH and c[Eaca¹,DNaI⁶]-GnRH) did not display the perfect sigmoidal IP stimulation exhibited by others; this was suggestive of a second low affinity binding site. The precise interaction of the ligand with the GnRHR is unknown and low-affinity interactions have been proposed in some tissues (Pahwa *et al.* 1991). It is also possible that this may reflect a non-specific interaction of a hydrophobic analogue at the very high dose (10^{-5} M) when this was observed.

Partial agonism is rare for GnRH analogues; in fact, this activity has largely been overlooked in the design of partial

agonists for GnRH, except for two reports emphasizing the critical role of His² in activating the GnRH receptor (Vale *et al.* 1972) and head to tail cyclization (Rivier *et al.* 1986) that results in a steric constraint that may prevent the analogue from assuming the proper conformation for full receptor activation. Whereas we have found that the length of a bridge used to restrain a particular conformation in GnRH antagonists is critical for maximization of potency *in vivo* or binding affinity *in vitro* (Rivier *et al.* 2000a, 2000b, 2000c), we see little effect of bridge size (from Gly to ε-aminocaproic acid) in the binding properties of the head to tail bridged analogues (Table 2). This suggests that those spacers are not part of the pharmacophore and that the common amino acids assume a similar conformation independently from the bridge.

Interestingly, analogues constrained by the introduction of a lactam ring (c(4–10)[Asp⁴,DNaI⁶,Dpr¹⁰]-GnRH and c(4–10)[Dpr⁴,DNaI⁶,Asp¹⁰]-GnRH) yielded full antagonists with both GnRHR systems. This is noteworthy since all other antagonists of GnRH have substitutions at any of the three positions 1–3. It is therefore conceivable to propose two different approaches to the design of GnRH antagonists based on functional/structural modifications that ultimately yielded analogues such as cetorelix, abarelix and degarelix. One approach is to introduce a lactam ring between the side chains of residues at positions 4 and 10 without modifying the pGlu-His-Trp N-terminus. In fact, we show here for the first time that full antagonists with significant binding affinity are generated while keeping the N-terminal pGlu-His-Trp moiety intact. This can best be explained as the result of a conformational constraint imposed onto the whole molecule. The finding that the potency of some of the peptides to inhibit binding of radiolabelled buserelin did not absolutely correlate with IP production should not be surprising since it has been consistently documented that for both small and large polypeptide hormones, receptor binding and signal transduction may be dissociable functions (Coy *et al.* 1982, Valove *et al.* 1994).

Recent substitutions attempting to improve the binding affinity of the partial agonists described here included modifications at positions 5 and 6 using some of the optimized substitutions used in the design of the latest generation of GnRH antagonists (Jiang *et al.* 2001). Those analogues are all full antagonists. This does not imply that such compounds cannot be designed. There is no doubt that potent partial agonists may be useful in clinical practice, since they may provoke significant suppression of the hypothalamic–gonadotroph axis and allow endogenous oestrogen to remain at the level needed for maintenance of bone density and prevention of symptoms of oestrogen deficiency. For example, Horvath *et al.* (2004) have approached this challenge using low doses of the GnRH antagonist, cetorelix. Their results suggest that cetorelix in low doses induces only a partial pituitary–gonadal inhibition and might be indicated for

treatment of endometriosis, leiomyomas and benign prostatic hyperplasia.

Whether the partial or the low-dose GnRH antagonist will prevail to obtain partial inhibition of the gonadal axis remains to be seen. It is conceivable that a partial agonist approach will lead to slow, in onset, but ultimately complete desensitization of the GnRHR, whereas the low dose of a GnRH antagonist will ultimately lead to early 'escape' and therefore to challenges in dosing.

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