Progress towards the development of non-peptide orally-active gonadotropin-releasing hormone (GnRH) antagonists: therapeutic implications

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Gonadotropin-releasing hormone (GnRH) is a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly.NH₂) which is produced from a precursor polypeptide in hypothalamic neurons and secreted in a pulsatile fashion to stimulate the secretion of LH and FSH via its interaction with a cognate receptor on gonadotropes¹,². Low doses of the native peptide delivered in a pulsatile manner to mimic that found in the hypothalamic portal vessels restore fertility in hypogonadal patients, and are also effective in treating cryptorchidism and delayed puberty²-⁴. Administration of high doses of GnRH, or agonist analogues, causes desensitization of the gonadotrope with consequent decline in gonadal gametogenesis and steroid and peptide hormone synthesis²⁴. This phenomenon finds extensive therapeutic application in clinical medicine in a wide spectrum of disease (Table 1)²-⁵. In addition, GnRH analogues have promise as new generation male and female contraceptives in conjunction with steroid hormone replacement⁶,⁷. GnRH antagonists inhibit the reproductive system through competition with endogenous GnRH for the receptor and, in view of their rapid effects, are being increasingly used for the above mentioned applications. The peptide agonists and antagonists currently available require parenteral administration, typically in the form of long-acting depots. A new generation of non-peptide GnRH antagonists are beginning to emerge which should allow oral administration and, therefore, may provide greater flexibility of dosing, lower costs and increased patient acceptance.

GnRH peptide analogues

A number of studies have indicated that the conserved -NH₂ and -COOH terminal domains of GnRH are closely apposed when mammalian GnRH binds its receptor and this is proposed to result from a β-II type turn involving residues 5-8 (Fig. 1). This is partly due to intramolecular...
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D-amino acid substitution enhances activity

Receptor binding and activation
D-amino acid substitution in antagonists

Receptor binding only

Fig. 1 Schematic representation of GnRH in the folded conformation in which it is bound to the GnRH pituitary receptor. The molecule is bent around the flexible glycine in position six. Substitution with D-amino acids in this position stabilises the folded conformation and decreases metabolic clearance. This feature is incorporated in all agonist and antagonist analogues. The –NH₂ and –COOH termini are involved in receptor binding. The –NH₂ terminus alone is involved in receptor activation and substitutions in this region produce antagonists. From Millar et al. with permission.

Table 1 Applications of GnRH and GnRH analogues

<table>
<thead>
<tr>
<th>Pulsatil GnRH (stimulation)</th>
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<tr>
<td>Infertility</td>
<td>stimulates gametogenesis and hormonogenesis</td>
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<td>Cryptorchidism</td>
<td>descent of testes</td>
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<td>Delayed puberty</td>
<td>advances puberty</td>
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<tr>
<th>GnRH agonists and antagonists (inhibition)</th>
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<tr>
<td>Contraception</td>
<td>Inhibition of ovulation and spermatogenesis with add-back sex steroid hormones</td>
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<td>Hormone dependent diseases</td>
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<tr>
<td>Prostatic cancer</td>
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<tr>
<td>Benign prostatic hypertrophy</td>
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<td>Breast cancer</td>
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<td>Endometriosis</td>
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<td>Uterine fibroids</td>
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<td>Premenstrual syndrome</td>
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<td>Polycystic ovarian syndrome</td>
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<td>Hirsutism</td>
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<td>Acne vulgaris</td>
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<td>Precocious puberty</td>
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<td>Acute intermittent porphyria</td>
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| Infertility | Inhibition of endogenous gonadotropin together with controlled administration of exogenous gonadotropin, especially in induction of ovulation in assisted reproduction techniques |
interactions with the side chain of Arg\(^8\), as various studies, including Trp fluorescence, computer simulations using the technique of conformational memories, and NMR, have shown that substitution of Arg\(^8\) (e.g. with Gln\(^8\) as in chicken GnRH I) results in a more extended structure and loss of predominance of the folded conformers (see reviews\(^8,9\)). Yet these extended forms (e.g. Gln\(^8\)GnRH) have high activity in many non-mammalian GnRH receptors. The ß-II type turn conformation of GnRH seems also to be induced or stabilised by the interaction of Arg\(^8\) with an acidic residue in extracellular loop 3 (EC3) of the mammalian receptor (see below). Substitution of a D-amino acid for Gly\(^6\) enhances the ß-II type conformation and increases the activity of Arg\(^8\) GnRH about 10-fold in mammals\(^8,9\). The D-amino acid substitution overcomes the deleterious effects of Arg\(^8\) substitution (e.g. with Gln\(^8\)) such that binding affinity for the mammalian receptor is increased almost 1000-fold\(^8\). Modification of the C terminal Gly\(^10\)NH\(_2\) also enhances activity, especially in combination with substitution of Gly\(^6\) with a D-amino acid\(^8,9\). Both of these modifications also decrease degradation of the peptide and are generally employed in both agonists and antagonists.

Modification of the conserved amino terminal residues (and especially the His\(^2\) residue) in GnRH resulted in analogues with antagonistic properties, as these residues are important in stabilising the receptor in the active conformation\(^8\). Overall, favourable substitutions have been identified at all positions in the native sequence. Replacement of His\(^2\) in combination with various modifications to enhance binding form the basis of all peptide antagonists thus far. Examples of GnRH agonists and antagonists employed in clinical practice are shown in Figure 2. The wide-spread use of these analogues in clinical medicine is reflected by current sales which exceed $2 billion per annum.

**Advantages and disadvantages of GnRH peptide analogues: the need for non-peptides**

While desensitization with GnRH agonists has revolutionised therapies of numerous diseases, there are two major disadvantages associated with their administration. Firstly, there is an undesirable initial stimulation of the reproductive system lasting several weeks before the onset of desensitization. This gives rise to a delay in ameliorative action and in some cases an initial flare in symptoms. The disadvantage of the initial stimulation by agonists is overcome by utilising GnRH antagonists. However, considerably higher doses are required; milligrams in contrast to micrograms per day. Secondly, these small peptides, like the agonists, are not orally active and have to be administered by injection or nasally. The disadvantage of daily injections has been addressed by the development of biodegradable
depot preparations which may last for several months. While these preparations ensure patient compliance, there are disadvantages of not being able to withdraw treatment when desired by the physician or patient and the inability to tailor dosages. There may also be adverse reactions at injection sites and the possibility of formation of antibodies which may cross-react with unknown natural proteins as well as the peptide itself.

Orally-active small molecule GnRH antagonists will potentially overcome a number of these disadvantages, increase therapeutic options and expand current therapeutic applications. Unlike peptide depots, a wide range of doses may be explored to allow partial suppression of gonadal steroids to levels sufficient to ameliorate disease (e.g. in endometriosis and uterine fibroids) without the side-effects of hypo-oestrogenism (e.g. bone loss)\(^\text{10}\). In view of their rapid onset of action and rapid cessation of action when no longer administered, oral antagonists might find application in short-term relief in various diseases such as bleeding from uterine fibroids and pain accompanying endometriosis. It is also likely that oral administration will be more acceptable to patients than daily injection or large implants.
The first cloning of the GnRH receptor from a mouse gonadotrope cell line\textsuperscript{11} revealed that it is a member of the large G-protein coupled receptor super family which is characterised by seven transmembrane (TM) domains connected by intracellular (IC) and extracellular (EC) domains. This facilitated the cloning of many other GnRH receptors from other species including the human receptor\textsuperscript{12,13}.

The availability of the cloned human receptor has allowed its stable expression in cell lines which have provided the means for high throughput screening for small molecule GnRH antagonists (see below) and for studies on the structure and ligand-binding of the receptor. A knowledge of the three-dimensional structure of the GnRH receptor is essential for a complete understanding of its molecular functioning. In the absence of physical studies (e.g. X-ray crystal structure), molecular models of the GnRH receptor have been generated on the basis of the limited knowledge of the physical structure of rhodopsin\textsuperscript{8,14-16}.

A molecular model of the GnRH receptor has been generated and has formed the basis for experimental studies designed to test hypotheses and refine the model\textsuperscript{8,16}. By site-directed mutagenesis, we have been able to validate and refine the model by establishing the orientation of the faces of TM helices 2 and 7, and the position of two disulphide bridges which anchor the N terminus and EC loops 1 and 2 (see review\textsuperscript{8}). The conservation of hydrophilic residues along distinct faces of the TMs in GnRH receptors spanning 500 million years of evolution\textsuperscript{17} has assisted in determining the orientation of these faces of the TMs towards the hydrophilic pocket formed by the helical bundle (Fig. 3).

Using this refined model and our knowledge of the functionally important residues in GnRH, various potential GnRH ligand contact sites have been proposed and tested by site-directed mutagenesis\textsuperscript{8}. A large number of amino acids in the EC domains and superficial regions of the TMs accessible to GnRH were mutated and studied for binding affinity, ligand-selectivity and efficacy. These revealed four putative GnRH contact sites. In the mouse receptor, Glu\textsuperscript{301} (Asp\textsuperscript{302} in human) is critical for receptor interaction with Arg\textsuperscript{8} of GnRH\textsuperscript{18}. The Gly\textsuperscript{10}, NH\textsubscript{2} appears to interact with Asn\textsuperscript{102} of the receptor\textsuperscript{19} and His\textsuperscript{2} and/or pGlu\textsuperscript{1} of GnRH appears to be involved in receptor activation through interaction with the Lys\textsuperscript{121} residue\textsuperscript{20}. His\textsuperscript{2} also seems to interact with the Asp\textsuperscript{98} residue\textsuperscript{21}. These interactions are schematically shown in Figure 3.

The contact sites of peptide antagonists have not been defined and are likely to differ from those of GnRH agonist\textsuperscript{20} although photoaffinity labelling of the receptor with an antagonist\textsuperscript{22} indicates that they bind in a site which overlaps that of the agonist.
Fig. 3 Schematic representation of GnRH interaction with the human GnRH receptor. The receptor is viewed from above and shows the transmembrane helices as a cluster of cylinders (going into the page) encompassing the hydrophilic pocket and surrounded by the hydrophobic membrane environment. The TM helices are connected by the extracellular loops. The two disulphide bridges between EC1 and EC2 and between EC2 and the N terminal domain are indicated by solid bars. GnRH is shown in its folded conformation in interacting via pGlu/His with Lys¹²²(K), His with Asp¹⁰⁴(D), Arg with Asp¹⁰⁴(D) and Gly¹⁰ NH₂ with Asn¹⁰¹(N) of the receptor. Modified from Millar et al² with permission.

The development of orally-active GnRH analogues

The insights on the GnRH binding pocket have indicated an approximate size for non-peptide molecules which could occupy this site and function as competitive antagonists. These are likely to approximate to the size and character of other GPCR mimetics such as the benzodiazepine, opiate and biogenic amine analogues. By stably transfecting the human GnRH receptor into cells with appropriate reporter genes (e.g. luciferase driven by the serum response element), or by direct receptor binding competition, it has been possible to screen large numbers of compounds. The lead
compounds identified may then be chemically modified using proven strategies of medicinal chemistry, but potentially also with the knowledge of the side chain properties of amino acids in the GnRH binding pocket of the receptor, to optimise their biological activity. For these molecules in which non-human GnRH receptors bind these analogues with an affinity several orders of magnitude less than that of the human GnRH receptor, chimaeras may be constructed to identify rapidly the domains involved in conveying high affinity binding of the non-peptide.

The emergence of non-peptide GnRH antagonists

Because of their commercial potential, the development of non-peptide GnRH antagonists has been the target of extensive efforts by the pharmaceutical industry. Therefore, most of the information about these molecules is to be found in the patent literature, although some reports are beginning to emerge in traditional scientific publications. A number of representative compounds are shown in Figure 4.

The first non-peptide GnRH antagonist appears to have been disclosed in a patent by Ho (McNeilab Inc.)\(^24\). This compound, (McN4923; 1 in Fig. 4), is a fused tetracyclic benzodiazepine which blocks ovulation in rats when given at a dose of 0.5 mg/kg.

In the scientific literature, the first publication of a non-peptide GnRH antagonist was by De and co-workers at Abbott\(^25\). The authors discovered that the antifungal drug ketoconazole (Nizoral, Janssen Pharmaceutica, Beerse, Belgium; 2 in Fig. 4) bound and inhibited the rat pituitary GnRH receptor with an apparent IC\(_{50}\) of 2 \(\mu\)M. Addition of a number of groups to this core structure, such as di- and tri-peptides related to GnRH, improved affinity slightly to approximately 500 nM.

The cloning and expression of the human GnRH receptor\(^{12,13}\), made screening of small molecule compound collections possible in order to identify lead molecules which bind the human receptor. This was followed by a series of patents from scientists at Takeda Pharmaceuticals describing benzodiazepines\(^26\) (e.g. 3 in Fig. 4), spiro-amines\(^27\) (e.g. 4 in Fig. 4) and thienopyridones\(^28\) (e.g. 5 in Fig. 4). Unlike peptide analogues, which have for the most part shown similar affinities for a variety of species, these small molecules can exhibit surprising species selectivity. For example, compound 4 binds the rat receptor with high affinity (IC\(_{50}\) = 9 nM) but binds the human with much lower affinity (IC\(_{50}\) = 400 nM). This trend was observed to a greater or lesser degree for the entire series of analogues for this compound reported. Conversely, compounds such as 5 are highly selective for human (IC\(_{50}\) = 0.2 nM) compared to 60 nM in the rat\(^28\). This low affinity for the rat receptor can invalidate convenient and inexpensive in vivo assays in the rat (or mouse), thus hindering drug development.
Furthermore, the 20-fold lower affinity reported for compound 5 binding to the cynomolgus monkey receptor, requires that even in vivo studies in primates must be carefully planned and interpreted in order to provide a meaningful model of interaction with the human receptor. Nevertheless, it is pertinent to recall that the clinically effective peptide analogues were all developed using rodent test models in spite of species differences in binding affinities of the peptides.
It is apparent from Figure 4, that a number of different companies are devoting substantial effort to the development of non-peptide GnRH antagonists. Takeda Pharmaceuticals has disclosed a number of different compound series (e.g. 2, 4, 5, and 9 in Fig. 4). Through a joint venture with Abbott, Takeda markets Lupron, the largest selling GnRH peptide agonist and, therefore, developing orally-active compounds has apparently been a high priority for their research efforts. Abbott (e.g. compound 1 in Fig. 4) was also one of the first companies to disclose non-peptide antagonists, and more recently reported the modification of erythromycin A derivatives to convert them into GnRH antagonists (8 in Fig. 4). Merck has described both quinolones (e.g. 7 in Fig. 4) and a series of indoles (e.g. 6 in Fig. 4) as GnRH antagonists. The most recent contribution in the field is Alanex Corp. (e.g. 10 in Fig. 4). It is quite likely that a number of other companies also have on-going efforts in this area, but the results of these efforts have not yet been made public.

Structure–activity relationships

The many different classes of molecules shown in Figure 4 together with the wide variety of peptide agonists and antagonists illustrate that high affinity binding to the GnRH receptor may be achieved using a broad range of chemical entities. It further suggests that it is not the particular arrangement of chemical bonds in any given scaffold which is critical for binding, but rather the placement of key molecular features in a particular 3-dimensional configuration (a pharmacophore) which is complementary to a cognate binding pocket in the receptor. Structure activity studies of peptides have shown that all residues in the peptide can contribute favourable binding interactions with the receptor, some of which have been mapped to contacts with specific amino acids in the receptor sequence (see above). It has been proposed that the non-peptides shown in Figure 4 may mimic subsets of these interactions. However, it should also be considered likely, that, in addition to mimicking peptide-receptor interactions, the small molecules could also establish additional novel interactions as has been shown to be the case for non-peptide antagonists of the substance-P and CRF receptors.

The hypothesis of corresponding features between non-peptide and peptide ligands was employed by Cho et al. to discover and optimize T-98475. Initially, a ‘directed screening’ approach was employed which focused their high through-put screening effort to compounds which were similar to known non-peptide ligands of other G-protein coupled receptors. This identified a heterocyclic compound which inhibited $^{[	ext{125I}]}	ext{-leuprorelin}$ binding by 67% at 20 µM concentrations. They hypothesized that the small molecule mimicked residues in the β-turn portion of GnRH, where the $p$-
methoxyphenyl and ester groups correspond to side-chains of Tyr^7 and Leu^7, respectively. Accordingly, a basic centre equivalent to Arg^8, found to be critical for peptide binding^8,9,18, was introduced into the small molecule screening lead component, with a concomitant increase in binding affinity (see below). Subsequent optimization of functional groups surrounding the bicyclic thienopyrimidone core resulted in T98475 with sub-nanomolar affinity for the human receptor. These findings indicate the value of understanding the peptide binding pocket in refining non-peptide lead compounds.

The presence of a basic group is a recurrent theme in both peptide and non-peptide GnRH antagonists^8,9. However, not all ligands require this feature as exemplified by compound 3 and the observation that most non-mammalian GnRHs lack the Arg^8 residue which is a crucial feature in the mammalian GnRH^8,9. It is also pertinent that the loss of affinity of GnRH in which Arg^8 has been substituted by a neutral amino acid can be overcome by the substitution of Gly^6 with a D-amino acid^18. In addition to a basic group, relatively subtle substitution patterns on an aromatic ring have been shown to have pronounced effects on affinities of both peptides (e.g. 4F DPhe^2)^9 and non-peptides^28 indicating that a tight interaction with the receptor is involved. However, there is no clear evidence of a correspondence between these rings in different compound series, and the possibility remains that they may interact with distinct sub-sites in the receptor. More extensive structure-activity studies and computational chemistry will be required to develop detailed pharmacophore models to reconcile these data.

The interactions of non-peptide GnRH antagonists with specific residues in the GnRH receptor remain to be elucidated. Based on molecular modelling studies, Cho et al^28 have put forward a model of the bound complex with T-98475. They propose that the basic nitrogen of T98475 engages in a favourable ionic interaction with Asp^302 which has previously been shown to interact with Arg^8 of mammalian GnRH^18. We have shown that mutation of Asp^302 to the neutral Asn does indeed reduce binding affinity of the analogue (Assefa et al, unpublished observations). Testing the binding characteristics of these small molecule antagonists to the mutant receptors which have been used to identify peptide-receptor interactions is likely to be a valuable tool in understanding how these compounds interact with the receptor and if, in fact, they mimic interactions utilized by the peptide ligands.

**Future directions**

It is likely that orally-active, non-peptide GnRH antagonists will become an important new class of therapeutics in coming years. However, numerous challenges remain. The patent and scientific literature reveals efforts that
Non-peptide orally-active GnRH antagonists are still in the early stages of the drug discovery process. As yet, non-peptide GnRH antagonists have not been tested in humans. Takeda's compound, T-98475, shows inhibition of plasma LH following oral administration in castrated cynomolgus monkeys at a dose of 60 mg/kg is a promising early finding. However, as with all drug development projects, it is likely that additional chemistry will be required to optimize pharmacokinetic and pharmacodynamic properties. Nevertheless, it is reasonable to anticipate that the new generation orally-active GnRH antagonists will find their way into the clinic in the next few years. The emergence of safe, orally-active GnRH antagonists will enable clinical researchers to explore a range of new treatment strategies, provide an important alternative to long-term depots or daily injections and are likely to have a major impact on the utility of GnRH analogues in an increasing range of treatment of human diseases.

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References

13 Kakar SS, Musgrove LC, Devor DC et al. Cloning, sequencing, and expression of human
gonadotropin releasing hormone (GnRH) receptor. *Biochem Biophys Res Commun* 1992; 189: 289-95
26 Ohkawa S, Fuji, N, Kato K, Miyamoto M. Condensed Heterocyclic Compounds, Their Production and Use as GnRH Antagonists. *WO 95/2990; 1995*
29 Sauer DR et al. 3′-N-Bis-Substituted Macrolide LHRH Antagonists. *WO 9950275, 1999*
31 Goulet M, Bugianese RL, Ashton WT et al Antagonists of Gonadotropin Releasing Hormone. *WO 97/21435; 1997*
32 Anderson M, Polinsky A, Hong Y, Gregor V. New Carbonylaminoalkyl Derivatives Are Gonadotropin Modulators – Useful for the Treatment of Sex Steroid-Dependent Disorders and Precocious Puberty. *WO 994498; 1999*