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Gonadotropin-Releasing Hormone Analog Design. Structure-Function Studies Toward the Development of Agonists and Antagonists: Rationale and Perspective

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Introduction

ON JUNE 24, 1971, Andrew V. Schally announced the determination of the primary structure of porcine GnRH at The Endocrine Society Meeting in San Francisco. This announcement was followed by publications by Matsuo *et al.* (1, 2) and Baba *et al.* (3) on the proposed amino acid sequence for porcine GnRH and its synthesis and by Burgus *et al.* (4) who characterized ovine GnRH and found the sequence to be identical with that of porcine GnRH. The physiological and therapeutic importance attributed to the discovery of the new substance was greatly increased by the prospect of the design of potent and long acting GnRH agonists and antagonists. Since that time more than 2000 analogs of GnRH have been synthesized. The impact of research of GnRH and its analogs on clinical medicine recently led Ziporyn (5) to note, "There's almost no subspecialty of medicine that will be left untouched by the [research] advances associated with LHRH or its analogs." It is the intent of this article to provide a historical review of the major, and some minor, aspects of the chemical development of GnRH agonists and antagonists up to the present state of development (July 1, 1985). The synthetic chemical efforts have been devoted largely to increasing the affinity of the peptides to the GnRH receptor and their resistance to degradation or elimination in *in vivo* systems, characteristics which, for the GnRH analogs, are generally interrelated.

An annual compilation and review of structure-activity relationships of GnRH analogs is available in the Specialist Periodical Reports which cover the literature published during 1971–1980 (volumes 4–13) (6–15). At irregular intervals, Annual Reports in Medicinal Chemistry furnish brief updates of the studies of structure-activity relationships of GnRH analogs (16–21). These reports, in conjunction with two recently published comprehensive monographs should provide the reader with a balanced account of the development of GnRH analogs (22–29).

Synthesis, Purification, and Characterization of GnRH Analogs

It is important to note that the rapid development of GnRH analogs was made possible through the extensive use of solid phase peptide synthesis (SPPS) introduced by Merrifield (30). As one of the codevelopers of the method (31), Stewart (32) has pointed out that the use of automated equipment for SPPS, benzhydrylamine-like resins for peptide amide synthesis (33, 34), and adequate methods for the purification of peptides, particularly reverse phase HPLC (RP-HPLC) in recent years (35), have made the synthesis of mammalian GnRH, <Glu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2, and its analogs, a relatively simple task. Although classical (solution) methods have been employed for the synthesis of GnRH (see Ref. 36 and references therein) and its analogs (37), it is quite clear that the use of SPPS and RP-HPLC were essential for the rapid exploration of structure-activity relationships as well as providing investigators with relatively large amounts of these substances for pharmacological, toxicological, and clinical studies.

While the purity of the agonists synthesized either by SPPS or classical (solution) methods was always of concern in terms of the concomitant biological activity of potential racemization products, it was a critical factor in the biological evaluation of the antagonists. This was particularly true in the early stages of development when the GnRH inhibitory activities were very low and could be masked by a small amount of racemized material

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present as a contaminant and acting as an agonist. For example, the high GnRH potency of [D-Ala^6]GnRH (350–450% of GnRH) would barely be affected by a 10% racemization contaminant of [L-Ala^6]GnRH (4% of GnRH) (38). However, a very weak antagonist, *e.g.* [D-Phe^2]GnRH, (39) could have its activity masked by the presence of small amounts of [L-Phe^2]GnRH (40) with only 2–4% of the potency of GnRH but with full intrinsic activity *in vitro*. The separation of the possible diastereomers, *e.g.* [D-Ala^6]GnRH and [L-Ala^6]GnRH, which could result from racemization, was eventually made feasible through the use of HPLC, thus eliminating one element of uncertainty from the interpretation of the biological results (35). Similarly, a preparation of [D-His^2]GnRH exhibiting 10% GnRH-like potency (39) was subsequently shown, when purified by HPLC, to be inactive either as an agonist or an antagonist (41). Racemization of histidine during peptide synthesis is well documented and difficult to prevent irrespective of whether classical methods of synthesis or SPPS are used. The widespread use of HPLC resulted from the recognition that classical methods of purification were inferior to HPLC as a tool for the separation of these diastereomeric peptides. Most GnRH analogs reported in the literature have been characterized by amino acid analysis only, often without a quantitative determination of the unnatural amino acids. Investigators have relied on the presumed authenticity of the starting protected amino acid and on high coupling efficiency during the assembling of the peptides on the resins rather than pursuing rigorous methods of characterization of the peptides. Nuclear magnetic resonance (NMR) and mass spectrometry were employed in those cases where definite proof of structure was required. Optical rotations were generally measured, and TLC and HPLC, in several systems, were used for proof of homogeniety.

Development of GnRH Agonists

The original incentive for the development of more potent GnRH agonists was the expectation that the knowledge of the LH-releasing and ovulation-inducing effects of GnRH observed in laboratory animals could be applied to the treatment of male and female infertility (42). However, the half-life of GnRH is very short (43, 44) and more potent and longer acting analogs were thought to be necessary for practical clinical utility, regardless of any anticipated or unanticipated therapeutic applications. Potent agonists, referred to as superagonists, were rapidly produced and were subsequently discovered, along with GnRH, to have, ironically, antireproductive effects. They were available for reproductive pharmacological evaluation (42, 45, 46) within 3 yr of the structure elucidation of GnRH; this accounts for their rapid clinical exploration exemplified by the first demonstration (in 1978) of inhibition of reproductive function in women by a superagonist (47). Once superagonists had been synthesized and their potential therapeutic value was recognized, further incentive, after 1976, to seek structurally novel and longer acting agonists was provided by promising commercial considerations.

The various biological assays and animal models that have been utilized for the testing of GnRH agonists have been recently reviewed (42, 48). The most widely used *in vitro* assays have been, initially, the dispersed pituitary cell for the measurement of LH and FSH secretions and, more recently, the receptor binding assay using purified pituitary membrane fractions for the estimation of the potencies of the analogs (49, 50). *In vivo* biological assays, which have been utilized to determine the potencies of GnRH agonists, include induction of ovulation, disruption of the estrus cycle, stimulation of uterine growth, inhibition of pregnancy, stimulation of LH release in ovariectomized rats, and stimulation of LH/FSH release in immature rats using an infusion technique (42, 48).

Pro^9-ethylamide (NEt) modifications

The first important structural modification of GnRH leading to increased potency was discovered by Fujino and co-workers (37), who examined the effect of structural modifications at the C terminus of GnRH. Although the des-amide of GnRH (GnRH free acid) exhibited very low GnRH potency in ovariectomized rats (51) and Pro^9-GnRH showed only 10% of the potency of GnRH *in vitro* (34), replacement of the glycine amide terminus with alkyl amines produced nonapeptide alkyl amides with significantly greater ovulation-inducing potency than GnRH itself (37). Thus, [Pro^9-ethylamide (NEt)]GnRH, the most potent analog of the series, was 5 times more potent than GnRH and more potent than either the Pro^9-methylamide (NHMe) or Pro^9-propylamide (NHPr) modifications. The fluorinated ethylamide analog, [Pro^9-NHCH$_2$CF$_3$]GnRH was subsequently reported by Coy *et al.* (52) to be twice as potent as [Pro^9-NEt]GnRH in releasing LH when administered to immature male rats. The data of Fujino *et al.* (37), it was noted, suggested that the terminal glycine amide was not essential for high potency and that the total chain length of the peptide played an important role in the binding affinity of the analog for the pituitary receptor. It was also suggested that the introduction of this Pro-alkylamide moiety may also increase the duration of action of these analogs by virtue of their greater resistance to enzymatic degradation (53). These two desired properties, greater binding affinity and enzymatic resistance to proteolysis, were to become the basis for the rational

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design and for the explanation of activity, or lack thereof, of all the GnRH analogs regardless of the site of structural modification. The concept that protection from renal elimination would also lead to prolonged action was eventually incorporated into the design of GnRH analogs.

D-Xaa^6 modifications

The second important structural modification of GnRH, discovered by Monahan *et al.* (38), was the replacement of the Gly^6 residue with D-alanine yielding [D-Ala^6]GnRH with a potency of approximately 350–450% that of GnRH both *in vitro* and in ovariectomized rats. The corresponding [L-Ala^6]GnRH had only 4% of the potency of GnRH, and it was suggested that since the potencies determined *in vivo* were in agreement with the *in vitro* results, it was unlikely that the differences in the biological activities could be solely explained by differences in clearance rates. Instead, the increased biological potency was attributed to the conformational stabilizing effect of the D-alanine which was favorable for binding (and activity) at the receptor. This study is also noteworthy for its suggestion that GnRH may conformationally contain a β-II type bend (involving Ser–Tyr–Gly–Leu) which is stabilized in [D-Ala^6]GnRH and preferable for binding at the receptor site. This point will be discussed below when the contribution of conformational studies to the design of GnRH analogs, particularly antagonists, is reviewed. Also to be deferred for later discussion is our knowledge of the enzymatic degradation of peptides and its contribution to the design (if any) of GnRH analogs. However, it should be noted that, regardless of whether the Gly^6–Leu^7 bond or the Tyr^5-Gly^6 bond is considered to be a major site of proteolytic cleavage, substitution of glycine by D-amino acids is likely to render either linkage more resistant to enzymatic degradation (26).

Additive effects (or lack thereof) of Pro^9-NEt and D-Xaa^6 modifications

It is often assumed that the biological effect of combining several structural changes in one molecule will be additive (or, more correctly, multiplicative). Thus, according to this additivity rule (54), if one structural modification leads to a relative potency of *a* and a second modification to a relative potency of *b* then the combination of both structural modifications in a single molecule would be expected to yield an analog with a biological potency of *a* multiplied by *b*. The potential additivity of biological potency of the Pro^9-NEt and D-Ala^6 modifications was immediately tested and, in rapid succession, two reports [Coy *et al.* (55) and Fujino *et al.* (56)] on this important combination appeared. Infusion experiments with immature male rats showed that the three analogs, [D-Ala^6,Pro^9-NEt]GnRH, [D-Ala^6]GnRH, and [Pro^9-NEt]GnRH, had LH/FSH releasing potency ratios of 12–16:7–8:2.5, respectively, compared with GnRH (55). Similar ovulation-inducing potency ratios were observed (56) among the three analogs, but the potencies relative to GnRH were much higher. These *in vivo* results correlated with the *in vitro* results of Vale *et al.* (57) who, using the stimulation of LH secretion from rat pituitary cells in culture by GnRH agonists as an index of potency, noted that the combination of the two structural modifications yielded an analog with a potency approximately equal to the product of the potencies of the individual modifications. The *in vitro* results of Fujino *et al.* (56) did not agree with these findings. Although it is self-evident that comparisons of biological data by different groups are valid only if the same bioassays are employed in precisely the same manner (48), nevertheless, many of the apparent disagreements in the data reported, regarding comparisons of potencies of superagonists, can be attributed to a disregard of this axiom. Thus, the validity of the extension of the additivity principle to the combination of a D-aromatic amino acid in position 6 and the Pro^9-NEt modification became a focal point of interest with the publication of apparently conflicting reports on this subject (57–59). The observation was made that agonists with D-aromatic amino acids such as [D-Trp^6]GnRH and [D-Trp^6,Pro^9-NEt]GnRH are much more potent (36 times and 144 times the *in vitro* potency of GnRH, respectively) than the corresponding substitutions with aliphatic amino acids, such as [D-Ala^6]LHRH and [D-Ala^6,Pro^9-NEt]GnRH, which are approximately 4 times and 14 times, respectively, the potency of GnRH (57). The subsequent binding studies of Perrin *et al.* (50) showed increased binding potencies for the Pro^9-NEt modifications as compared with [D-Ala^6]GnRH or [D-Trp^6]GnRH; however, when compared with the biological potencies, in stimulating LH secretion *in vitro*, the increases were far less dramatic. The data for the D-Trp^6 analogs are in accord with the subsequent binding studies of Barron *et al.* (60) but are not in accord with the earlier *in vitro* data reported by Coy *et al.* (58). *In vivo* measurements in immature rats comparing [D-Leu^6]GnRH and [D-Leu^6,Pro^9-NEt]GnRH (Leuprolide), using integrated levels of LH over a 6-h period after injection, showed a similar additive effect (61), but the additive effect was reported not to hold true for the corresponding D-aromatic amino acid modifications in this same assay system (59). Thus, in male rats, the Pro^9-NEt modification was reported to decrease the potency of [D-Phe^6]GnRH and [D-Trp^6]GnRH by a factor of nearly 2 (59). However, it has been more recently reported that, using estrus suppression (62) as an index of agonist activity, [D-Trp^6]GnRH and [D-Trp^6,Pro^9-NEt]

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GnRH were equipotent. Postcoital comparisons in rats (Naqvi, R. and M. Lindberg, unpublished observations) also indicated that the two analogs were equipotent. In women, it was reported that the two analogs were equipotent with respect to the sc dose required for maximal LH release (63). Barron *et al.* (60) showed that MCRs in pregnant women were similar for both analogs. They concluded that since the NEt residue, which is reported to protect the peptide from postproline-cleaving enzyme activity, did not lead to a prolonged survival time in pregnant women, degradation by this enzyme in human tissues contributes minimally to GnRH clearance. Support for this conclusion was found in reports that both analogs were equipotent *in vivo* (42, 64). Thus, the overwhelming evidence points to *in vivo* equipotency for the two D-Trp^6 analogs irrespective of the *in vitro* results and binding studies supporting the additive effects on the biological potency of the D-Trp^6 and Pro^9-NEt modifications.

Hydrophobic modifications at position 6

The trend toward seeking more potent agonists with increasing hydrophobic character resulted in the addition of two more superagonists to the growing list of analogs available for clinical exploration. [N^τ-Bzl-D-His^6,Pro^9-NEt]GnRH (Histerelin) was designed by Rivier *et al.* (22) to have the characteristics of high water solubility at acidic pH and greater lipophilic character *in vivo*, while retaining high biological potency. A correlation was noted between the *in vitro* potencies of certain position 6 superagonists and their HPLC retention times at physiological pH. These correlations included [N$^\tau$-Bzl-D-His^6,Pro^9-NEt]GnRH and [D-Trp^6,Pro^9-NEt]GnRH, with the former being slightly more potent than the latter, *in vitro. In vivo* results showed a similar trend (48).

It had previously been observed that the incorporation of D-amino acids, with larger and more lipophilic side chains than in [D-Leu^6]GnRH, such as [D-Trp^6]GnRH, resulted in more potent agonists (57, 59). Nadasdi and Medzihradszky (65) proposed a quantitative correlation to exist between the potency of position 6 substituted GnRH analogs and the calculated hydrophobicity of the amino acid side chain. It is accepted that increased lipophilicity of drugs is generally associated with greater retention of the drug in the body and, therefore, prolonged duration of action (66). The retention may be the result of enhanced renal reabsorbtion or fat storage of nonionized fat-soluble compounds. Protection of the drug from renal excretion, through plasma protein binding, will also affect its duration of action. Plasma protein binding generally increases, in a given series of analogs, with increasing hydrophobicity (66). With this in mind, Nestor *et al.* (62, 67) postulated that analogs with greater hydrophobicity could have an extended biological half-life resulting from a whole body depot effect. They would attribute this effect to a decreased rate of clearance of the analog from the circulation and the increased binding capacity of the analog for hydrophobic plasma carrier proteins (67). The results of the study on a wide range of hydrophobic analogs showed that the most potent ones were found in a hydrophobicity range, as measured by their retention time on RP-HPLC (68), greater than that of the D-Trp^6 analogs. As an example, [D-Nal(2)^6]GnRH (Nafarelin acetate), the most potent of this series, was reported to be 200 times more potent than GnRH in suppressing estrus in rats. It was pointed out, however, that analogs with greater hydrophobicity than Nafarelin acetate were less potent and this includes the analog incorporating the Pro^9-NEt modification into Nafarelin acetate (62). Interestingly, [D-Nal(2)^6]GnRH and [D-Nal(1)^6]GnRH were isolipophilic but the latter was 4-fold less potent. Other examples (68, 69) also bear witness to the inadequacy of using hydrophobicity alone as a prediction of agonist potency (28).

Nafarelin acetate, which was reported to be twice as potent as [D-Trp^6]GnRH, [D-Trp^6,Pro^9-NEt]GnRH, or [N^τ-Bzl-D-His^6,Pro^9-NEt]GnRH in estrus suppression comparisons (26), became the last superagonist to be made available for clinical exploration. The improvement of the pharmacokinetics with the more hydrophobic agonist may appear to be supported by the comparisons of the reported half-lives of GnRH [$t_{1/2}$ = 8 min, constant infusion, (44)], [D-Trp^6]GnRH [$t_{1/2} \simeq$ 30 min, constant infusion, (44)], and [D-Nal(2)^6]GnRH [$t_{1/2}$ = 2.4 h, sc administration, (67)]; however, comparison of the three peptides under identical conditions is not available. The considerably longer plasma elimination half-lives reported for Nafarelin acetate in rats, monkeys, and humans than reported for GnRH or [D-Leu^6,Pro^9-NEt]GnRH (Leuprolide) were attributed, at least in part, to the more extensive plasma binding of Nafarelin acetate (70).

N^ω,$N^{\omega'}$-dialkyl-D-homoarginines were incorporated into position 6 of GnRH agonists (67, 71) as a result of successful GnRH antagonist investigations with this unnatural amino acid. The most potent, [N^ω,$N^{\omega'}$-diethyl-D-Har^6,Pro^9-NEt]GnRH, was only slightly less potent than [D-Nal(2)^6]GnRH in the rat estrus suppression assay.

Other C-terminally modified analogs

Another structural modification which has generally led to increases in potency, in combination with D-amino acids in position 6, is the α-aza-Gly^{10}(-NHNHCO-) substitution. A series of α-aza analogs of GnRH were syn-

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thesized by Dutta *et al.* (72, 73) with the expectation that the presence of an α-aza residue might be conformationally favorable, leading to higher binding affinity, and be more resistant to enzymatic degradation. Replacement of amino acids in position 6, 9, and 10 of GnRH by α-aza amino acids alone did not confer any potency advantage but when the α-aza-Gly^{10} modification was combined with, for example, the D-Ser(Bu^t)^6 substitution, the resulting analog, [D-Ser(Bu^t)^6, α-aza-Gly^{10}]GnRH, currently undergoing clinical development, was considered to be at least 5 times more potent than [D-Ser(Bu^t)^6,Pro^9-NEt]GnRH, Buserelin, (74), using induction of ovulation as a measurement of potency (72, 73). (Buserelin has been, clinically, the most extensively studied GnRH analog.) It was unclear, to Dutta *et al.*, which individual factor was primarily responsible for this enhancement of biological potency. [2-D-Nal^6, α-aza-Gly^{10}] GnRH was reported to be slightly more potent than Nafarelin acetate and approximately 2.5 times more potent than the corresponding Pro^9-NEt modification in the estrus suppression assay (26). However, Nestor (26) noted that if the α-aza-Gly^{10} substitution conferred high potency by virtue of its enhanced resistance to the post-proline-cleaving enzyme in rat plasma, then the relevance of this substitution to human therapy was less clear since the amount of postproline-cleaving enzyme present in human plasma was reported to be 2–5 times less than in rat plasma (75, 76). Does the statement of Nestor also imply that any C-terminal amide modifications generally would not confer any advantage over the parent Gly^{10}-NH$_2$ function in humans? On the basis of the human data available on [D-Trp^6]GnRH and [D-Trp^6,Pro^9-NEt]GnRH (60, 63, 64), the answer would appear to be yes, although systematic comparisons would have to be made.

Position 7 modifications

[*N*-Me-Leu^7]GnRH was found to be equipotent with GnRH and [D-Ala^6,*N*-Me-Leu^7]GnRH was found to be at least as active as [D-Ala^6]GnRH *in vitro* (77). In fact, the *N*-Me-Leu^7 modification has been incorporated into [D-Trp^6,Pro^9-NEt]GnRH yielding [D-Trp^6,*N*-Me-Leu^7,Pro^9-NEt]GnRH, an analog currently undergoing clinical development (63). Generally, the effect of the *N*-Me-Leu^7 modification in enhancing the potency of the parent peptide depends on the D-amino acid at position 6 and the bioassay used to compare their potencies (45). The introduction of the bulky alkyl side chain, the tert-butyl group, as an ether into serine, which proved to be successful in significantly enhancing potency when incorporated into the 6-position (*e.g.* Buserelin), also enhanced potency at the 7-position, of nonapeptide NEt analogs of GnRH (74). The combination of two D-Ser(Bu^t) groups at position 6 and 7 not only failed to enhance, but actually decreased, the ovulation-inducing potency of Buserelin.

Conformationally constrained and backbone modifications

With the recognition that the biological activity of [D-Ala^6, *N*-Me-Leu^7]GnRH was consistent with that of a β-turn conformation for residues 5–8 of GnRH, Freidinger *et al.* (78) introduced a γ-lactam as a conformational constraint into the 6,7 position of GnRH and found that the resulting analog was 9 times more potent than GnRH *in vitro* and, by iv injection in ovariectomized rats, 2.4 times more potent than GnRH. Further exploration of the γ-lactam modification has not been made with agonists, *per se*, but this modification has been tried with GnRH antagonists. Various attempts to obtain a conformationally restricted agonist through cyclic analog design have yielded inactive analogs (79) or agonists with low biological potency (22, 80). Spatola (81) has reviewed the effect of peptide backbone modification on structure-activity relationships. Backbone modifications, as new approaches to GnRH agonist design, resulted in relatively little *in vitro* potency in the cases of peptide bond reversals at the 5–6 or 5–6 and 6–7 position (retro-inverso analogs) (82) or in the cases of substitution of a thiomethylene (-CH$_2$S-) group for the peptide linkage at the 5–6, 6–7, or 9–10 position of GnRH. The latter substitution at 9–10 had 10% of the *in vitro* potency compared to that of GnRH, indicating the necessity of more precise conformational requirements for residues 5–8 than for residues 9–10 (23).

Before closing the discussion on the current stage of development of the GnRH agonists, it is necessary to comment upon efforts to increase the potency of the agonists by modifying other amino acid residues.

Miscellaneous modifications

The 1-L-Nal^3 substitution (83) in GnRH is the only modification in position 3 which resulted in a peptide with greater potency than GnRH (twice as potent). Interestingly, [2-L-Nal^3]GnRH (84) was only half as potent as GnRH in stimulating LH release in ovariectomized rats. Although substitutions at the other remaining positions, 1, 2, 4, 5, 8, and 9, by naturally or unnaturally occurring amino acids, have resulted in decreased potency relative to GnRH, none of these residues are fundamentally required for GnRH activity. Each of these residues can be substituted to give active analogs, albeit with reduced potency. Fragments of GnRH and truncated (deletion) analogs of GnRH possessed very low GnRH potency. A series of peptides successively shortened from the C-terminus of GnRH were essentially inactive, except for Pro^9-GnRH which had 10% of the

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potency of GnRH *in vitro* (34). A concise review of these very early explorations is given by Coy *et al.* (85), and an extensive compilation is offered in the earlier volumes of the Specialist Periodical Reports cited above.

Modifications of naturally occurring vertebrate GnRH

The sequences of salmon GnRH with Trp^7,Leu^8 substitutions (86), chicken I GnRH with a Gln^8 substitution (87–89), and chicken II GnRH with His^5,Trp^7,Tyr^8 substitutions (90), relative to mammalian GnRH, have been recently discovered. Salmon GnRH and chicken I GnRH were isolated using RIAs based on antibodies raised against mammalian GnRH. Chicken II GnRH, with three drastic changes in a portion of the molecule considered important for receptor binding/activation, was isolated on the basis of its ability to release LH in an *in vitro* dispersed cell culture assay. The high relative potency of chicken II GnRH (30% that of mammalian GnRH) was unexpected in view of the results obtained with salmon and chicken I GnRH, which were only 1–2% as potent as mammalian GnRH in this same assay. The lead brought about by this observation, that [His^5,Trp^7,Tyr^8]GnRH (or chicken II GnRH) can still result in an analog with high *in vitro* relative potency, has been, so far, largely unexplored. Preliminary studies including substitution by D-amino acids at position 6 of some of the naturally occurring nonmammalian GnRH peptides have yielded analogs that are more potent than the parent peptides (91, 92), suggesting a secondary structure similar to that of GnRH. The Pro^9-NEt modification has also been introduced in conjunction with a D-amino acid at position 6 into nonmammalian GnRH (92). On the basis of preliminary biological studies conducted in goldfish, however, Peter *et al.* (92) concluded that the structural modifications that determine superactivity of mammalian, chicken, and salmon GnRHs in goldfish differ from what is known for mammals. Finally, a frog brain GnRH was partially characterized, and its structure was proposed to be identical with that of GnRH on the basis of the HPLC behavior of the isolated material and its immunological characteristics in several RIAs (93, 94).

Enzyme Degradation Studies

The literature on enzymatic degradation of GnRH and its analogs has been recently reviewed by Flouret *et al.* (95). The recognition of the enzymatic instability of GnRH as the reason for its relatively short lived biological effect prompted early half-life studies with GnRH. However, the discovery of the stabilizing effect of the position 6 modification (whether enzymatic, or conformational and resulting in greater binding affinity) preceded any attempts to experimentally determine the enzymatic cleavage sites of GnRH or its analogs. Since the high potency of the [D-Xaa^6]GnRH agonists had been rapidly realized, there was little practical incentive to design more potent agonists based on subsequent metabolic studies. It may be for this reason only that Flouret *et al.* (95) can legitimately claim: "The design of active LHRH analogs has not been guided by metabolic studies, but rather metabolic studies have been used to rationalize the high biological activity of some analogs." Certainly knowledge of the actual enzymatic cleavage sites was used to design some early antagonists (96). However, if this statement of Flouret *et al.* is historically valid, then it is also true that the retrospective rationalization has been highly speculative and, not necessarily, physiologically relevant. There is sufficient disparity between the *in vivo* and *in vitro* enzymatic results so as to lead Flouret *et al.* (95) to also conclude: "Attempts to correlate analog potency with resistance to degradation by tissue homogenates or by purified tissue peptidases must be cautiously evaluated, as these enzymes probably are compartmentalized and might not come in contact with LHRH or its analogs under physiological conditions."

It was originally reported by Koch *et al.* (97), using rat hypothalamic extracts, that Gly^6-Leu^7 was a major cleavage site of GnRH. A study by Marks and Stern (98), using rat brain homogenates, implicated Tyr^5-Gly^6 and Pro^9-Gly^{10}, in addition to Gly^6-Leu^7, as cleavage sites. Koch *et al.* (99) correlated the increased resistance of [D-Ala^6]GnRH and [D-Trp^6]GnRH to rat hypothalamus and anterior pituitary GnRH degrading endopeptidases with increased biological potency. Bauer and co-workers found <Glu^1-His^2 (100), Tyr^5-Gly^6 (and His^2-Trp^3, from the resulting N-terminal pentapeptide) (101), and Pro^9-Gly^{10} (102) as major cleavage sites of GnRH but not Gly^6-Leu^7, using purified enzymes isolated from bovine brain and pituitary homogenates. Thus, *in vitro*, three enzymes were identified: pyroglutamate aminopeptidase, endopeptidase, and postproline-cleaving enzyme. However, the relevance of these *in vitro* findings in contrast to the *in vivo* results, where <Glu^1-His^2 (43), His^2-Trp^3, Trp^3-Ser^4, and Ser^4-Tyr^5 (95) have been identified as cleavage sites of GnRH, is unclear.

Horsthemke *et al.* (103) concluded that even though superagonists modified at positions 6 and 10 are more resistant than GnRH to enzymatic degradation, there was no strict colinearity between their enhanced agonist activity and their resistance to degradation. Therefore, it was noted, factors other than resistance to degradation, such as increased receptor binding or stimulation, must be considered as being responsible for the potency of the superagonists. They also obtained evidence that substituents at positions remote from the enzymatic site of attack affect the cleavage rates. More recently, Flouret

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et al. (104) have conducted renal tissue studies with [D-Ser^4]GnRH, [D-Trp^6]GnRH, and [D-Ser^4,D-Trp^6]GnRH. It was found that [D-Ser^4]GnRH blocked enzymatic cleavage at position 4 but then Gly^6-Leu^7 was revealed as an ancillary scissile site. [D-Trp^6]GnRH, similarly, was not cleaved at position 6, nor, surprisingly, at the Ser^4-Tyr^5 bond, but was cleaved at the His^2-Trp^3 and Trp^3-Ser^4 bonds. Finally, [D-Ser^4,D-Trp^6]GnRH was degraded *in vivo* by proximal tubules at the His^2-Trp^3 bond. Flouret *et al.* (104) concluded that their data demonstrate important inhibitory effects of D-amino acid substituents remote from the scissile peptide bond. In fact, however, their data also indicate the general vulnerability of peptidic linkages, unprotected by multiple D-amino acids, to enzymatic degradation by various tissue peptidases.

Clinical Explorations

Some of the superagonists currently undergoing clinical trials or development have been identified above. The following is a summary: [D-Leu^6,Pro^9-NEt]GnRH (Abbott Laboratories, North Chicago, IL); [D-Trp^6,Pro^9-NEt]GnRH (Salk Institute, La Jolla, CA); [D-Trp^6] GnRH (Tulane University, New Orleans, LA); [D-Trp^6,*N*-Me-Leu^7, Pro^9-NEt]GnRH (Wyeth Laboratories, Philadelphia, PA); [D-Ser(Bu^t)^6,Pro^9-NEt]GnRH (Hoechst AG, Frankfurt, West Germany); [D-Ser(Bu^t)^6, α-aza-Gly^{10}]GnRH (Imperial Chemical Industries, Macclesfield, Cheshire, England); [N^τ-Bzl-His^6,Pro^9-NEt] GnRH (Salk Institute); [D-Nal(2)^6]GnRH (Syntex Laboratories, Inc., Palo Alto, CA). All of these superagonists have D-amino acids at position 6. Some are also modified at position 10, incorporating the Pro^9-NEt modification, and one has the α-aza-Gly^{10} replacement. One analog incorporates a *N*-Me-Leu^7 substitution into the combined modifications at positions 6 and 10. Leupron (Leuprolide) is now available in the United States for use in the treatment of prostatic cancer. All other analogs are in various stages of clinical exploration and/or development both in academia and industry.

Rationale for Development of GnRH Antagonists

The original impetus for the development of the superagonists was the treatment of infertility and, only subsequently, were the antireproductive effects discovered. However, the rationale for the development of the antagonists was derived from the immediate recognition that a competitive antagonist of GnRH had the potential of being a nonsteroidal contraceptive agent (105). In fact, a specific and generally confined physiological action was expected of the antagonists (106) which were intended primarily for female contraception (42). These analogs were expected to be free of the liabilities and/or toxicities associated with either of the components of the estrogen-progestagen combination pill. The greater safety anticipated for a metabolically and rapidly degradable peptidic contraceptive was another attractive feature of GnRH analogs even though it was recognized that if the analogs were to act as competitive antagonists to endogenous GnRH they would have to be continuously present at the receptor site in order to be efficacious. One approach would have been to generate irreversible inhibitors of GnRH; such inhibitors, however, were rejected as possible candidates due to their anticipated toxic and receptor-altering properties.

Animal Models/Biological Assay Correlations

The various biological assays employed for the testing of both agonists and antagonists have recently been reviewed (42, 48). With the discovery of the relatively potent disubstituted GnRH antagonists and the successful demonstration, for the first time, that an antagonist can suppress the preovulatory proestrus surge in the normal (4-day) cycling rat and thereby inhibit ovulation, a simple animal model became available for *in vivo* testing of GnRH antagonists (106). Other previously employed rat (107) and hamster models (108) were eventually abandoned for the routine testing of antagonists.

Corbin and Beattie (106) discussed the various biological models employed (pituitary cells; ovariectomized, steroid-blocked rats, *etc.*) and concluded that data translated from those models to the normal cycling female had to be employed with caution. Yardley *et al.* (109) noted that the *in vitro* and *in vivo* potencies of the analog pair, (des-His^2[D-Ala^6]GnRH and its Pro^9-NEt modification), were nonparallel and concluded that, by itself, antagonism of GnRH-induced LH secretion in cell cultures is inadequate in predicting antiovulatory activity. In contradistinction, Beattie *et al.* (110) demonstrated that potent antiovulatory analogs suppressed the proestrus, preovulatory LH surge, thereby clearly suggesting that this represented the basis for their antiovulatory activity. Later, with the availability of more potent antagonists, there was no longer a question of whether *in vitro* activity was generally predictive (110), qualitatively, of antiovulatory activity, but rather the question of whether there was a quantitative correlation between *in vitro* and antiovulatory activity in the rat. Bowers *et al.* (25) had concluded that for most of the antagonists, the *in vitro* and *in vivo* potencies were closely parallel. The most potent inhibitors of ovulation were always very potent *in vitro*, although there were instances of other antagonists possessing *in vitro* potencies identical with those of the potent ovulation inhibitors, but with little or no antiovulatory activity even at much higher doses (111). It was also concluded that quantitative and consistent estimates of antagonist potency could be obtained

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in vitro using either intact pituitaries of 21-day-old immature female rats (111) or dispersed pituitary cell cultures (112); however, potencies of antagonists are consistently much greater when they are assayed by the dispersed cell method than by the intact pituitary method with respect to the antagonist-GnRH dosage ratio as an index of antagonist potency.

It is accepted that the initial action of GnRH is to bind to specific receptors on the surface of its target cells. From a comparison of labeled GnRH agonists or antagonists, Perrin *et al.* (113) concluded that (1) similar kinetic and equilibrium behavior were displayed by agonists and antagonists and (2) the relative binding affinities of agonists and antagonists were not significantly different from one another irrespective of which radioligand was used in the radioreceptor assays. They further concluded that antagonists bound competitively to the same receptor sites available to agonists. They cited additional support for this latter conclusion from a report by Conn *et al.* (114), who showed that an antagonist can exhibit agonist properties when the antagonist is capable of causing receptor microaggregation. Photoaffinity studies with an antagonist led Hazum and Keinan (115) to the same conclusion: GnRH agonists and antagonists bind to the same receptor. This was consistent with their previous binding studies (116). Clayton and Catt (49) and Perrin *et al.* (50) noted that there was a general positive correlation between receptor binding affinity and relative *in vitro* antagonist activity.

More relevant, however, is the observation that the potency rankings of the antagonists depend upon the type of bioassay used (117). Receptor affinity represents only one parameter, and pharmacokinetic factors such as absorption, distribution, resistance to degradation, and elimination (excretion and/or metabolism of intact drug from plasma) also influence the *in vivo* efficacy of the analogs. Ultimately, the following question must be asked: "Is the rat antiovulatory assay, which has been the standard *in vivo* screening assay used, a predictive model for suppression of gonadotropins and for antifertility activity in the human female?" A similar question may be asked of the male rat as a predictor of efficacy in the human male. These questions will no doubt be discussed, in part, by other contributors to this volume. Nevertheless, is is important to note that antagonists, possessing a wide spectrum of potencies in rats, have indeed shown varying degrees of gonadotropin suppression in nonhuman primates as well as humans (see Table 2 under *Clinical explorations*). Finally, one anticipated advantage of the antagonists is the lack of an initial stimulation of gonadotropin release inherent in the GnRH agonists which may be undesirable in the use of agonists as contraceptive agents. Although little is certain concerning the possible uses of potent and long acting antagonists of GnRH, one can anticipate that they will be tested in all the clinical situations presently employed for GnRH superagonists.

Although the antiovulatory effects of the GnRH antagonists will be emphasized in this review, other antireproductive effects in female animals have been reported and recently reviewed (42, 118–120). Similarly, in the male, the gonadotropin suppression properties of the antagonists have been described (121) and recently reviewed (120, 122–124). Species differences have also been reported (125).

Development of GnRH Antagonists

Before it was even established that an antagonist could be developed, some doubts were expressed that the GnRH antagonist program of the Center for Population Research would have any more success than prior, partially successful efforts to develop other peptide antagonists (*e.g.* angiotensin, vasopressin, glucagon). The dramatic and rapid potency increases that were observed during the development of the GnRH agonists were not to be realized with the antagonists even when some of the structural features of the superagonists were incorporated early into the design of antagonists. The development of GnRH antagonists, with its requirement of high affinity for the GnRH receptor (without intrinsic activity) and resistance to enzymatic degradation, was slow and notable for the small, incremental increases in potency and by potency plateaus, as represented by a family of equipotent GnRH antagonists, which periodically appeared.

Early modifications: positions 2; 2,6; and 2,3,6

The first competitive antagonist of GnRH to be reported was des-His^2-GnRH by Vale *et al.* (126) using the dispersed pituitary cell culture assay. While [Gly^2]GnRH had partial agonist activity, des-His^2-GnRH showed no agonist activity at the doses tested, and both analogs reduced the secretion rate of LH in dispersed rat pituitary cells, as stimulated by GnRH, at molar ratios of 1,000 to 10,000 times that of GnRH. Explanations suggesting that the presence of His^2 was required, for recognition by the receptor or for GnRH activity, were clearly inadequate since [Phe^2]GnRH had 2–4% of the potency of GnRH, and [Trp^2]GnRH was 40% as potent *in vitro* (40). des-His^2-GnRH acts as a weak competitive inhibitor of GnRH because it lacks any functionality at position 2, which is required for some intrinsic activity, but still retains a structure with sufficient topological features remaining for recognition by the receptor. In this regard, both des-<Glu^1-GnRH and des-(<Glu^1-His^2)-GnRH showed very low, if any, GnRH agonist activity in rat pituitary tissue cultures (127). Other dele-

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tion analogs also showed very low agonist potency with the exception of Pro^9-GnRH which showed an *in vitro* potency of 10% that of GnRH (128). Thus, des-His^2-GnRH appears to be the only deletion analog reported to have antagonist activity in the absence of any other structural changes. Claims of lack of *in vivo* antagonist activity (129) for des-His^2-GnRH were attributed to its low intrinsic GnRH activity. This claim was refuted by a counter-demonstration of antagonist activity for des-His^2-GnRH in normal male rats (130) as well as equipotency with des-His^2[Pro^9-NEt]GnRH. The latter had been claimed by Coy *et al.* (129) to be the first inhibitor of GnRH found to be active *in vivo* (in ovariectomized and in estrogen- and progesterone-treated rats); subsequently, this inhibitor was reported to block ovulation induced by GnRH (131). Monahan *et al.* (38), utilizing the observations of *in vitro* antagonist activity for des-His^2-GnRH and high agonist activity for [D-Ala^6]GnRH, synthesized des-His^2[D-Ala^6]GnRH, which had 3 times the antagonist potency of des-His^2-GnRH. Monahan *et al.* (132) were the first to demonstrate that a D-amino acid substitution, *e.g.* [D-Ala^2]GnRH, would lead to a GnRH partial antagonist. However, a breakthrough (reported by Yardley *et al.* and Corbin and Beattie) resulted from combining the structural features of the weakly active antagonist, [D-Phe^2]GnRH (39), with those of [D-Ala^6]GnRH, yielding [D-Phe^2,D-Ala^6]GnRH, which was the first antagonist to inhibit ovulation (at 6 × 1 mg doses in a corn oil vehicle injected sc) in the normal cycling rat (106, 109). Other modifications at position 6, such as [D-Phe^2,D-Phe^6]GnRH, did not improve antiovulatory potency, but [4-F-D-Phe^2,D-Ala^6]GnRH (110) was the first example of the use of a halogenated phenylalanine residue in position 2, a substitution which would later play a very important role in greatly increasing antagonist potency. Incorporating the Pro^9-NEt modification into [D-Phe^2,D-Ala^6]GnRH yielded an antagonist which had *in vitro* activity (109), but lacked antiovulatory activity even at 10 times the dose of the parent, [D-Phe^2,D-Ala^6]GnRH. des-His^2[D-Ala^6,Pro^9-NEt]GnRH was also found to be less potent than des-His^2[D-Ala^6]GnRH *in vivo* and this was attributed to the higher inherent GnRH agonistic activity of the former (133). The Pro^9-NEt modification, even for the later, very potent antagonists, generally reduced rather than improved potency, suggesting that improved *in vitro* potency in the agonist series may be due to a stabilized secondary structure of the active conformer involving a dipole-dipole interaction of the NEt functionality with the imidazole ring of histidine. This folding would be analogous to that suggested in the case of TRH by Donzel *et al.* (134). The only reported all L- disubstituted antagonist, [Leu^2,Leu^3]GnRH, had very weak *in vitro* potency (135). The D-Ala^6 modification, [Leu^2,Leu^3,D-Ala^6]GnRH also possessed weak *in vitro* potency (136). However, by incorporating an aromatic L-amino acid into position 3 of [D-Phe^2,D-Phe^6]GnRH, Coy *et al.* (137) and de la Cruz *et al.* (138) found that the resulting analog, [D-Phe^2,Phe^3,D-Phe^6]GnRH, improved potency 2-fold, exhibiting complete antiovulatory activity at 3 × 1 mg doses. Similarly, Humphries *et al.* (139) reported that incorporation of L-proline into position 3 of [D-Phe^2,D-Trp^6]GnRH yielded [D-Phe^2,Pro^3,D-Trp^6]GnRH, which completely inhibited ovulation at a single dose of 750 μg. The apparent discrepancy in the antiovulatory and *in vitro* potencies of di- and trisubstituted analogs, *e.g.* [D-Phe^2,D-Phe^6]GnRH [inhibitory dose ratio ((IDR_{50}) = 23/1)] and [D-Phe^2,Pro^3,D-Trp^6]GnRH (IDR_{50} = 48/1), was subsequently attributed by Rivier and Vale and coworkers (140, 141) to greater resistance to degradation or to a decrease in residual intrinsic activity resulting from the presence of the position 3 modification. [D-Phe^2,D-Trp^3,D-Phe^6]GnRH, with three D-amino acid residues, showed 83% inhibition of ovulation with a single dose of 1 mg while N^{ϵ}-pentapeptidyl-Lys^6 derivatives of [D-Phe^2,D-Trp^3,D-Lys^6]GnRH were, on a molar basis, slightly more potent than the parent D-Phe^6 analog (142). Dutta *et al.* (72) combined the α-aza-Gly^{10} modification, previously used to increase agonist potency (72, 73), with [D-Phe^2,D-Phe^6]GnRH to give [D-Phe^2,D-Phe^6, α-aza-Gly^{10}]GnRH, which showed an 8-fold increase in antiovulatory potency, compared with the parent antagonist, using inhibition of GnRH-induced ovulation as an index of potency. No comparisons were reported using the normal cycling rat. The α-aza-Gly^{10} moiety was utilized occasionally in subsequent, potent antagonists, but its success in improving potency was limited and usually erratic (143).

Conformation studies: position 1 modifications

The octapeptide, des-His^2[D-<Glu^1,Pro^9-NEt]GnRH was prepared specifically with the anticipation that an N-terminal D-amino acid would decrease the rate of degradation of the enzyme (96), presumably, pyroglutamate aminopeptidase (100, 144). The analog had some antagonist activity in ovariectomized rats, but the successful utilization of a D-<Glu^1 substitution was not realized until the next major breakthrough occurred.

Until this time, progress in increasing the potency of the GnRH analogs had been based largely on the utilization of classical functional group modifications of the side chains of GnRH coupled with changes in chain length of the peptide. The concept of conformational stabilization of a β-II type bend involving Ser-Tyr-Gly-Leu residues of GnRH had been invoked to explain the high potency of [D-Ala^6]GnRH relative to GnRH itself (38). The first attempt to introduce a conformational

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constraint into a GnRH agonist, based on the premise of Monahan *et al.* (38) and a test of the use of conformation-activity relationships for the rational design of GnRH analogs, was reported by Donzel *et al.* (79) who synthesized [Glu^4,D-Ala^6,Orn^7]GnRH. The biological inactivity of both the cyclic and linear analog was attributed to the differences in the side chains of Glu^4 and Orn^7 as compared with those of Ser^4 and Leu^7 (in GnRH). Molecular models were used to represent peptide conformations such as that of GnRH (145, 146), but, because of the inherent flexibility in peptide bonds, the use of such models is unenlightening in the absence of any other structural information (38). Spectroscopic studies employing NMR (147, 148) and optical rotary dispersion/circular dichroism (149, 150) led to the conclusion that GnRH behaves as a random coil in water and is devoid of any intrachain residue interactions (150). Marche *et al.* (149) noted a trend toward ordered structures upon increasing the temperature at pH 7.4 while Mabrey and Klotz (150) observed that GnRH exhibited a conformational transition in trifluoroethanol with the formation of a β-structure which may be relevant to biological activity at the receptor site. Momany (151–153) was the first to employ semiempirical conformational energy calculations on GnRH and its analogs. Since it was not feasible to examine all of the possible conformations of GnRH, Momany (151) restricted his calculations by utilizing 1) the observation concerning the conformational stabilization resulting from the introduction of D-Ala^6 into GnRH (38), 2) a ^{13}C-NMR study showing that the proline peptide bond, in GnRH, was completely trans (154) and 3) the "computationally derived low energy 'dipeptide' conformations of the residues involved." He concluded, by energy minimization techniques, that there were three low energy conformations of GnRH, one of which appeared to explain the observed analog data: the so-called CC conformer with the N- and C-terminus in close proximity, as speculated by Grant and Vale (146). He also noted that the low energy structures had a modified type II bend involving Tyr-Gly-Leu-Arg rather than Ser-Tyr-Gly-Leu as proposed by Monahan *et al.* (38). These observations, supported by the subsequent work of Freidinger *et al.* (78), would now explain why [D-Ala^6,*N*-Me-Leu^7]GnRH had enhanced and not reduced biological potency as originally predicted (77). Leu^7 is not involved with Ser^4 in hydrogen bonding in this modified β-II type bend, and, therefore, *N*-Me-Leu^7 would not disrupt the modified β-II type bend. In spite of the limitations of Momany's study (the use of semiempirical calculations to determine preferred conformations and the inability to identify a single global minimum amongst the low energy conformers), a working model with which to test further structure-activity relationships of GnRH analogs was provided.

Rivier and Vale (140) proceeded to use, as a rationale for introducing D-<Glu^1 in position 1, Momany's observation that the analogs containing L-<Glu^1 and D-amino acids at positions 2, 6, and/or 3 had low energy conformers with a common configurational property. The *cis* peptide bond of the L-<Glu ring in these antagonists had changed orientation with respect to that of the L-<Glu ring in the CC conformer of GnRH. Reversing this situation, by replacing L-<Glu^1 with D-<Glu^1 in the antagonists, would reorient the *cis* peptide bond of the D-<Glu ring in the antagonists to coincide with the orientation of the L-<Glu ring found in the CC conformer of GnRH. Although the analog suggested by Momany [D-<Glu^1,Phe^2,D-Xaa^6]GnRH, was not synthesized, the corresponding D-Phe^2 trisubstituted analog [D-<Glu^1,D-Phe^2,D-Phe^6]GnRH (155) was prepared. This analog lacked antiovulatory activity at the dose tested (750 μg) but did exhibit *in vitro* antagonist activity. However, when D-<Glu^1 was combined with [D-Phe^2,D-Trp^3,D-Trp^6]GnRH by Rivier and Vale (140), the resulting antagonist was found to have complete antiovulatory activity at 250 μg/rat and to exhibit *in vitro* potency in a molar ratio of 3:1 with GnRH. This was a 5-fold increase in antagonist potency with respect to the parent analog *in vitro*. Other D-<Glu^1 analogs were less potent or showed no advantage over the prototype, *in vitro* or *in vivo* (140). [D-<Glu^1,D-Phe^2,D-Trp^3,D-Trp^6]GnRH also demonstrated prolonged activity in the antiovulatory assay, possibly due to greater enzymatic resistance *in vivo* or to delayed absorption when suspended in corn oil. It was the first antagonist reported to totally block ovulation when administered as a single injection at 0900 h on the day of proestrus. The N^{ϵ}-isophthaloyl dimer of [D-<Glu^1,D-Phe^2,D-Trp^3,D-Lys^6]GnRH (156) completely inhibited ovulation at 250 μg; it had 6 times the potency of its monomeric parent. The use of cyclopentane carboxylic acid in position 1, as suggested by Momany (153), was unsuccessful, *in vivo* (157). It should also be noted that, even with the evidence for the modified β-II type bend, the conformation of GnRH or its analogs was still largely unknown. It was proposed that it was more likely that GnRH and its analogs exist as an ensemble of conformers in solution rather than a single conformer (158). Subsequent NMR studies (159) supported the modified β-turn involving Tyr-Gly-Leu-Arg. Some empirical evidence for the occurrence of the CC conformation in solution was offered by NMR and circular dichroism measurements (160), but under no circumstances could this evidence be considered as proof. However, the accumulated evidence supports the β-turn, or the folding involving Tyr-Gly-Leu-Arg, in GnRH, as the bioactive conformation in this region (78, 159). X-ray studies on GnRH and its analogs have been hampered

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by the difficulty encountered by crystallographers in growing appropriate crystals necessary for such studies.

4-Cl(and F)-D-Phe² modifications: increasing hydrophobicity

The pace of discovery of new potent antagonists quickened. Humphries *et al.* (161) showed that Ac-Pro¹ could replace D-<Glu¹ with comparable antiovulatory potency for [Ac-Pro¹,D-Phe²,D-Trp³,⁶]GnRH, emphasizing that a D-amino acid in position 1 was not essential and, in fact, the corresponding Ac-D-Pro¹ analog was less potent than either the D-<Glu¹ or the Ac-Pro¹ modification. Channabasavaiah and Stewart (162) extended the range of position 1 acetyl-D-amino acids that could be accommodated in the antagonist structure to Ac-D-Ala and Ac-D-aromatic amino acids, in general, with antiovulatory activity in the 100–250 μg range. The necessity of the acetyl group for high potency in this case was realized when it was subsequently shown (156) that [D-Phe¹,D-Phe²,D-Trp³,D-Phe⁶]GnRH was inactive at 1 mg/rat. However, with the additional discovery by Coy *et al.* (156) that the use of 4-Cl-D-Phe in position 2 dramatically increased the antiovulatory potency of this peptide (82% inhibition at 250 μg), the exact electronic requirements (carbonyl *vs.* aromaticity) at position 1 were unclear. Nevertheless, combining 4-Cl-D-Phe² with Ac-D-Phe¹ yielded [Ac-D-Phe¹,4-Cl-D-Phe²,D-Trp³,⁶]GnRH which exhibited complete antiovulatory activity at 62 μg in a propylene glycol-saline vehicle and significant inhibition of ovulation at 15 μg in a corn oil vehicle (possibly due to prolongation of release of the antagonist from the corn oil medium). With the increasing hydrophobic alignment of aromatic D-amino acids at positions 2,3, and 6, Spatola *et al.* (163) found that even the nonbulky Ac-Gly¹ provided a potent antagonist, [Ac-Gly¹,4-Cl-D-Phe²,D-Trp³,⁶]GnRH, with 100% antiovulatory activity at 25 μg in a corn oil vehicle. Exploration by Rivier *et al.* (22, 68) of the parent analog [D-Phe²,D-Trp³,⁶]GnRH led to the conclusion that [Ac-Δ³-Pro¹,D-Phe²,D-Trp³,⁶]GnRH, containing the acetyl-3,4-dehydroproline¹ residue, was significantly more potent than other position 1 substituents. When this modification was then combined with 4-Cl-D-Phe², the resulting analog, [Ac-Δ³-Pro¹,4-Cl-D-Phe²,D-Trp³,⁶]GnRH, showed complete inhibition of ovulation at 7.5 μg in corn oil (22, 68).

Predictive and deductive approaches

A more systematic attempt was then made to improve potency by applying the manual method of Topliss (164) to the Hansch approach of drug design (22, 68). Since the apparent correlation of biological potency of certain GnRH superagonists with their overall hydrophobic character, as measured by HPLC, did not hold for a series of GnRH antagonists, it was concluded that the receptor requirements for recognition and binding may involve other factors in addition to hydrophobicity, such as localized electronic density and steric effects of substituents. The Topliss method offered an opportunity to identify these essential parameters, found in Hansch-type correlations, for loci which contain aromatic side chains. This method was applied to a series of [Ac-Δ³-Pro¹,Xaa²,D-Trp³,⁶]GnRH analogs. Although one analog was discovered (4-Br-D-Phe²) with higher *in vitro* potency, the classically identified 4-Cl-D-Phe² and the closely related 4-F-D-Phe² analogs were the most potent *in vivo*. Using the Topliss approach, the investigators were unable to identify a unique set of operative parameters, as many of the antagonists turned out to have similar potencies. It is likely that this approach, which has seen considerable success in small, rigid systems, is inapplicable to the much more flexible and larger peptide systems where biological potency resulting from electronic and steric changes in the molecule may be influenced by other parameters including conformational effects (Gierasch, L. M., private communication). Still further *in vivo*, but not *in vitro*, improvements were made by incorporating β-(2-naphthyl)-D-alanine into positions 3 and 6, which resulted in analogs such as [Ac-Δ³-Pro¹,4-F-D-Phe²,2-D-Nal³,⁶]GnRH. This analog completely inhibited ovulation at a dose of 2.5 μg (22). Previously, this D-amino acid had been used to obtain a potent agonist (62). Investigation of a series of hydrophobic antagonists, using 2-D-Nal in positions 3 and/or 6, led to the conclusion that Ac-Pro¹ could be substituted for Ac-Δ³-Pro¹, resulting in analogs with equipotency in an antiovulatory assay as measured by an ED_{50} rather than an ED_{100} (28). The *N*-Me-Leu⁷ modification did not significantly improve the potency of the Ac-Δ³-Pro¹ modification (22) nor did additional forays into modifications of position 5 using Topliss' manual method (27).

D-Ala¹⁰ modification

The use of D-Ala¹⁰ by Erchegyi *et al.* (165) resulted in the potent analog, [Ac-4-Cl-D-Phe¹,4-Cl-D-Phe²,D-Trp³,D-Phe⁶,D-Ala¹⁰]GnRH, which blocked ovulation in seven of eight rats at 7.5 μg. This analog is of interest since it contains five D-amino acid residues, which may confer enzyme resistance to peptide bonds throughout much of the sequence, except at the Ser⁴-Tyr⁵ and Leu⁷-Arg⁸-Pro⁹ portions of the peptide. The introduction of sterically larger groups, such as D-Ser¹⁰ and D-Leu¹⁰, resulted in less potent analogs, implying, perhaps, that side chain branching at position 10 hinders receptor binding. This D-Ala¹⁰ analog was the most potent analog found in a classical structure-activity study dealing particularly with positions 1 and 2 (165, 166). Administra-

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tion of analogs, including the early disubstituted ones, to rats before diestrus (106) or to immature male rats (167) allowed for the determination of duration of action of the antagonists *in vivo*. With the observation of prolonged activity with the potent tetrasubstituted analog (140), increased attention was turned to evaluating analogs for prolonged activity. Several Ac-Δ^3-Pro^1 analogs, examined by Rivier *et al.* (118) as well as the above D-Ala^{10} analog, examined by Coy *et al.* (24), showed extended duration of action in the animal models employed.

D-Arg^6 modification: efforts to increase hydrophilicity of antagonists

The rapid development of the GnRH antagonists was, by now, being accompanied by numerous residue changes, and it was necessary to frequently reevaluate individual amino acid substitutions relative to the rest of the molecule in order to optimize potency. With this in mind, and, in order to improve water solubility by the introduction of more hydrophilic amino acids, position 6 was reexamined in relation to the Ac-4-Cl-D-Phe^1 and D-Ala^{10} modification by Coy *et al.* (168). With their introduction of basic D-amino acids, such as D-Arg^6, the next breakthrough was realized. The most potent analog reported was [Ac-4-Cl-D-Phe^1,4-Cl-D-Phe^2,D-Trp^3,D-Arg^6,D-Ala^{10}]GnRH, with 100% inhibition of ovulation in the 1.5- to 3-μg range in corn oil and somewhat less potent in propylene glycol-saline. The observed difference in potency in the two vehicles was suggested (168) to be due to delayed and/or prolonged absorption of this D-Arg^6 antagonist. The corresponding tetrasubstituted analog, lacking D-Ala^{10}, was reported to be 10-fold less potent. The corresponding D-Lys^6,D-Ala^{10} analog was less potent than the D-Arg^6,D-Ala^{10} modification. Extension of this observation by Horvath *et al.* (169) showed that the Ac-2-D-Nal^1 pentasubstituted derivative, [Ac-2-D-Nal^1,4-Cl-D-Phe^2,D-Trp^3,D-Arg^6,D-Ala^{10}]GnRH, had approximately 3 times the potency of the corresponding Ac-4-Cl-D-Phe^1 analog. This study, and the need to frequently reevaluate other positions each time a significant single positional improvement is discovered in order to optimize potency, strongly indicates that there is still a large empirical component to GnRH analog design.

The hydrophilic tetrasubstituted antagonist, [Ac-Δ^3-Pro^1,4-F-D-Phe^2,D-Trp^3,D-Lys^6]GnRH, exhibited very high binding affinity (113), but no antiovulatory data were reported for this analog. However, the corresponding D-Arg^6 analog with comparable binding affinity showed unexpectedly low antiovulatory potency: 60% inhibition of ovulation at 20 μg in corn oil (27). This finding was similar to that of Coy *et al.* (168) who had previously synthesized and tested [Ac-Δ^3-Pro^1,4-Cl-D-Phe^2,D-Trp^3,D-Arg^6]GnRH and found no antiovulatory activity at 7.5 μg in propylene glycol-saline. However, high *in vivo* potency could be restored to the tetrasubstituted D-Arg^6 analogs (without D-Ala^{10}) by the introduction of the very hydrophobic residue, 2-D-Nal, at position 1. Thus, [Ac-2-D-Nal^1,4-F-D-Phe^2,D-Trp^3,D-Arg^6]GnRH (27) was reported to have complete antiovulatory activity at 1 μg in corn oil. Rivier *et al.* (27) concluded that, generally, the GnRH receptor will tolerate either a hydrophobic or a hydrophilic residue at position 6, provided that it is concomitantly paired with a hydrophilic (Ac-Δ^3-Pro) or hydrophobic (Ac-2-D-Nal) residue, respectively, at position 1. Coy and Nekola (170) arrived at the same conclusion on the basis of independent data. Commenting on the hydrophilic-hydrophobic surface model, proposed by one of us (M.J.K.), Coy and Nekola (170) observed that the modifications to the hydrophilic part of the peptide chain (or looped structure stabilized by the position 6 D-amino acid) often must be accompanied by suitable alterations in the complimentary hydrophobic region in order to maintain or increase antagonist potency. It is the alteration of the global properties of these peptides, sometimes in a very subtle manner, that has enabled investigators to achieve antiovulatory potency in the nanogram range. It should be noted that while the referenced hydrophilic-hydrophobic surface model of the antagonist is consonant with many of the past structure-activity studies, the model is no doubt an oversimplification which has already been contradicted by a subsequent study of Hocart *et al.* (171). One would have predicted, on the basis of this model, that position 7 could accommodate hydrophilic amino acids. However, the introduction of Lys or Thr, at position 7, was detrimental to potency, while Phe^7, a hydrophobic residue, improved potency (171). Thus, the model is not compatible with these biological results.

[Ac-2-D-Nal^1,4-F-D-Phe^2,D-Trp^3,D-Arg^6]GnRH was tested for duration of action and required 100 μg when given on diestrus II, as compared with 1 μg when given on proestrus, for complete inhibition of ovulation (27). Nekola and Coy (172) had shown that a 100 μg dose of [Ac-2-D-Nal^1,4-Cl-D-Phe^2,D-Trp^3,D-Arg^6,D-Ala^{10}]GnRH suppressed LH levels for more than 30 h in ovariectomized rats. [Ac-2-D-Nal^1,4-F-D-Phe^2,D-Trp^3,D-Arg^6]GnRH also had 100% oral antiovulatory potency at 2.5 mg (27). Nekola *et al.* (173) had previously shown that [Ac-4-Cl-D-Phe^1,4-Cl-D-Phe^2,D-Trp^3,D-Arg^6,D-Ala^{10}]GnRH was completely effective, at 2.0 mg, orally, in blocking ovulation. The low oral antiovulatory potency (after gavage) of these analogs as compared with their sc potency was attributed, by both groups of investigators, to poor oral absorption (<1% of the absorption rate by injection), enzymatic degradation, or elimination. Structure-activity relationships (based on potencies obtained by oral *vs.* sc administration) do not necessarily correlate

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(172), but it has been generally observed that the oral-sc potency ratio is about 1000:1 under the conditions tested.

Alterations at the C terminus, either by use of the Pro^9-NEt modification (27) or by α-aza-Gly^{10} (143), in conjunction with the D-Arg^6 or the 2-D-Nal^6 antagonist series, were usually, but not always (28, 173a), detrimental to improving biological potency. The *in vitro* or *in vivo* enhancements of potency that were seen in the GnRH agonist series by use of Pro^9-NEt or α-aza-Gly^{10} were rarely realized during the development of the antagonists.

The idea of combining basicity, aromaticity, and hydrophilicity into a single amino acid led Folkers *et al.* to introduce the heterocyclic amino acids, β-(3-pyridyl)-alanine (174) and β-(3-quinolyl)-alanine (175), into a series of GnRH antagonists at positions 3 and/or 6. These amino acids are less basic than arginine but have the aromatic properties of tryptophane and naphthylalanine. The most potent analog, [Ac-2-D-Nal^1,4-Cl-D-Phe^2,3-D-Pal^3,D-Arg^6,D-Ala^{10}]GnRH, was reported to completely inhibit ovulation at 500 ng, suggesting greater potency than the corresponding D-Trp^3 analog (169). Simultaneous use of 3-D-Pal at position 3 and 6 decreased potency with respect to the parent D-Trp^3,D-Arg^6 analogs. β-(3-Quinolyl)-D-alanine offered a comparable replacement for the D-Trp^6 antagonists but not for the D-Arg^6 antagonists in terms of biological potency.

In order to further test the predictions of conformation based on minimum energy calculations (153) Roeske and Anantharamaiah (176) imposed conformational constraints at the N terminus with the introduction of α-methyl-D-amino acids at positions 2 and/or 3. The biological results supported the proposed helical type III bend for position 2 substitutions, but the use of α-methyl-D-amino acids at position 3 caused considerable loss of biological potency compared with the unmethylated parent analogs. Rivier *et al.* (22) had previously found that introduction of α-Me-4-Cl-D-Phe^2 was only marginally detrimental to biological potency when compared with the corresponding unmethylated analog. Roeske and Anantharamaiah extended the use of α-methyl amino acids at position 2 to the more potent 3-D-Pal^3,D-Arg^6 antagonists. The resulting analog [Ac-2-D-Nal^1,α-Me-4-Cl-D-Phe^2,3-D-Pal^3,D-Arg^6,D-Ala^{10}]GnRH, was completely active in inhibiting ovulation at 500 ng (Roeske, R., N. Chaturvedi, and T. Hrinyo, unpublished observations).

N^ω,$N^{\omega\prime}$-Dialkyl-D-Har^6 modifications

Nestor *et al.* (62, 67) applied their concept of the importance of the depot effect to GnRH antagonists (28, 67, 177). A prolonged biological half-life is more critical for antagonists than agonists since effective competition with endogenous GnRH pulses demands the continuous presence of the antagonist at the pituitary receptor site. They noted that [Ac-4-Cl-D-Phe^1,4-Cl-D-Phe^2,D-Trp^3,D-Arg^6,D-Ala^{10}]GnRH (168), which contains the hydrophilic D-Arg^6 moiety, had a prolonged duration of action compared with [Ac-Pro^1,4-F-D-Phe^2,2-D-Nal^{3,6}]GnRH. They suggested that the prolonged activity of the D-Arg^6 analog may be due to a depot effect involving an electrostatic interaction between the negatively charged phosphate group of the phospholipid membrane and the positively charged guanidine group of D-Arg. [It may be appropriate to cite a model proposed by Schwyzer *et al.* (178) wherein the lipid phase of the target cell membrane initially interacts with the peptide and induces secondary structures and topological arrangements, which then facilitate receptor-peptide interaction.] To further stabilize the hypothesized interactions, a series of N^ω,$N^{\omega\prime}$-dialkyl-D-arginine and homoarginine residues were incorporated into GnRH antagonists. These residues, it was suggested, would have the potential for both electrostatic and hydrophobic interaction with the phospholipid bilayer. The most potent pentasubstituted analog in this series [Ac-2-D-Nal^1,4-Cl-D-Phe^2,D-Trp^3,N^ω,$N^{\omega\prime}$-diethyl-D-Har^6,D-Ala^{10}]GnRH, was compared, biologically, with some literature standards but not with the more closely related Ac-2-D-Nal^1 standard [Ac-2-D-Nal^1,4-Cl-D-Phe^2,D-Trp^3,D-Arg^6,D-Ala^{10}]GnRH (169, 172), which contains, as the only change, D-Arg^6 rather than N^ω,$N^{\omega\prime}$-diethyl-Har^6. In the only two structurally analogous comparisons with a D-Arg^6 analog, an Ac-4-Cl-D-Phe^1,N^ω,$N^{\omega\prime}$-diethyl-D-Har^6 analog was slightly more potent, in an antiovulatory assay, when the analog was administered on diestrus II (rather than on proestrus), than the corresponding D-Arg^6 analog and [Ac-2-D-Nal^1,4-F-D-Phe^2,D-Trp^3,N^ω,$N^{\omega\prime}$-diethyl-D-Har^6]GnRH was approximately 2.5 times more potent on diestrus II than the corresponding D-Arg analog (67). These results suggest, in the two comparisons cited, that the N^ω,$N^{\omega\prime}$-diethyl-D-homoarginine substitution for D-Arg in position 6 does not significantly increase acute biological potency but may, according to the investigators, substantially increase potency in terms of duration of action. However, it should be noted that there was a greater difference in potency, in terms of duration of action, between [Ac-2-D-Nal^1,4-Cl-D-Phe^2,D-Trp^3,N^ω,$N^{\omega\prime}$-diethyl-Har^6]GnRH (ED_{50} = 2.5 μg on diestrus II) and the corresponding Ac-4-Cl-D-Phe^1 analog (ED_{50} = 16 μg on diestrus II) than there was between the former analog and [Ac-2-D-Nal^1,4-F-D-Phe^2,D-Trp^3,D-Arg^6]GnRH (ED_{50} = 9 μg on diestrus II), which lacks the N^ω,$N^{\omega\prime}$-diethyl-Har^6 modification.

Position 7 modifications

Folkers *et al.* (179) used, as a rationale for further substitutions into the more potent antagonist, the ob-

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served differences between mammalian GnRH, salmon GnRH (86), chicken I GnRH (87–89), and chicken II GnRH (90). The four vertebrate GnRH structures were postulated (179) to have similar receptors, with minor structural changes enabling the different GnRH structures to bind to the different parent receptors. Thus, it was suggested (179) that the mammalian GnRH receptor might be able to bind an antagonist with changes in the 5, 8, and particularly in the 7 position, the attractive feature of the position 7 modification being that chicken II and salmon GnRH have Trp^7 substitutions. Milton *et al.* (180), however, had noted that their results suggested that there was a difference between the chicken receptor and the mammalian GnRH receptor in the recognition of GnRH analogs substituted in position 8. Nevertheless, the most potent analog of the series [Ac-2-D-Nal^1,4-Cl-D-Phe^2,3-D-Pal^3,D-Arg^6,Trp^7,D-Ala^{10}]GnRH was reported to show complete inhibition of ovulation at 500 ng and 90% inhibition (2/20 rats ovulated) at 250 ng, whereas the parent antagonist (without Trp^7) showed complete inhibition at 500 ng but only partial inhibition (3/7 rats ovulated) at 250 ng (179). A variety of other hydrophobic and aromatic basic amino acid substitutions at position 7 proved less potent. The combined substitution of Trp^7 with Trp^5, Phe^5, or 3-Pal^5 similarly showed no improvement in antagonist potency. Rivier *et al.* (181) and Hocart *et al.* (171) synthesized some position 7 modifications and obtained somewhat different biological results. Rivier *et al.* (181) reported that [Ac-2-D-Nal^1,4-Cl-D-Phe^2,D-Trp^3,D-Arg^6,Trp^7,D-Ala^{10}] GnRH, which contains the naturally occurring Trp^7 of fish GnRH (86), was equipotent (ED_{100} = 2.5 μg) with the corresponding Met^7 and Phe^7 in the antiovulatory assay using corn oil as a vehicle. Hocart *et al.* (171) approached position 7 modifications from another point of view, specifically, as a test of the hydrophilic-hydrophobic surface model (170). When hydrophilic substitutions at position 7 showed at least 2-fold less potency, and Phe^7 showed greater potency, than the parent analog in antiovulatory assays using 40% propylene glycol-saline as a vehicle, a variety of aromatic amino acid substitutions were made. The most potent, [Ac-2-D-Nal^1,4-Cl-D-Phe^2,D-Trp^3,D-Arg^6,Phe^7,D-Ala^{10}]GnRH (identical to that of Rivier *et al.* above) was reported to be approximately twice as potent (64% inhibition of ovulation at 0.5 μg) as the Tyr^7 antagonist and more potent than either the corresponding Trp^7 or 2-Nal^7 analogs. Hocart *et al.* concluded that the results indicate a preference for hydrophobic residues at position 7, but that beyond a certain point, increasing hydrophobicity has little effect on antagonist potency.

Transposition modifications involving positions 5 and 6

A transposition involving the introduction of a basic amino acid into position 5 was reported by Roeske *et al.* (182). They had considered interchanging the Arg^5,D-Tyr^6 residues for the Tyr^5,D-Arg^6 analog, based upon the Momany model of GnRH wherein residues 5, 6, and 7 have their side chains oriented along the outside of the modified β-II type bend. Similar to previous suggestions (67, 178), was the proposal that these side chains may not be in contact with specific groups in the receptor, but possibly interact nonspecifically with components of the cell membrane. [Ac-2-D-Nal^1,4-Cl-D-Phe^2,D-Trp^3,Arg^5,D-Tyr^6,D-Ala^{10}]GnRH completely inhibited ovulation at 2.5 μg or at approximately twice the dose of the parent Tyr^5,D-Arg^6 analog. The $N^{\omega},N^{\omega'}$-diethyl-Har^5,D-Tyr^6 and the N^{ϵ}-isopropyl-Lys^5,D-Tyr^6 analogs were somewhat more potent, showing almost complete inhibition (1/10 rats ovulated) at 1.0 μg. However, the corresponding Glu^5,D-Arg^6 analog was impotent at 10 μg. These results were interpreted as evidence in support of the view that the position 5 side chain interacts with the cell membrane.

Chain length and backbone modifications

References to shorter chain GnRH antagonists such as the Pro^9-NEt modifications and their general inapplicability to improvements in potency have already been given. One longer chain analog, an undecapeptide, endo-Pro^{1a}[D-Phe^2,D-Trp^{3,6}]GnRH (183), was equipotent with its parent Ac-Pro^1 analog, but this example is not necessarily a satisfactory test of the effect of chain length on antiovulatory potency, since the corresponding endo-Gly^{1a} and the Gly^1,endo-Pro^{1a} analogs were less potent. A number of pseudodipeptide backbone modifications of antagonists were made by Spatola *et al.* (23) at position 1–2, 6–7, and 9–10 using —CH_2S— (a methylene sulfide bond) to replace the peptide bond. As was previously found for the agonists (23), the least potent analogs *in vitro* had replacements at the 6–7 position, and it was concluded that such replacements lead to undue flexibility, whereas currently acceptable conformational models demand stabilization of a β-turn involving residues 6 and 7. Replacements at the 1–2 and 9–10 positions resulted in analogs which were equipotent with their parent peptides *in vitro*, but were impotent in antiovulatory assays at the doses tested. This lack of *in vivo* potency was attributed to impaired absorption in cases wherein the analog structures were beyond optimal solubility in the Hansch formulation (184). The sulfoxide replacement, —CH_2SO—, although more polar than —CH_2S—, did not remedy the situation. The methyl-substituted, chiral bond amide replacements, —$CHCH_3S$—, proved to be the most successful of the sulfur-based amide bond replacements, with *in vitro* potencies equivalent to the parent peptide bond analogs though they were still considerably less potent *in vivo* than anticipated.

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Cyclic antagonists: computer-assisted analog design

Early attempts to impose cyclic constraints on GnRH analogs led to agonists with relatively low potency or no activity at the doses tested (22, 79, 80). Nevertheless, the results of Seprodi *et al.* (80) were interesting in view of the fact that the N- to C-terminal cyclic analogs, cyclo[-β-Ala^1,D-Ala^6,Gly^{10}-]GnRH and cyclo[-6-aminohexanoic acid^1,D-Ala^6,Gly^{10}-]GnRH, having 1.2% and 0.65% of the potency of GnRH, respectively, *in vivo*, were more potent than the corresponding linear analogs. The corresponding 2-D-Nal^6 analogs exhibited 60% and 20% intrinsic activity, respectively, when tested *in vitro* and exhibited high binding affinity, [dissociation constant (K_d) = 5 and 0.5 mM as compared to K_d = 5 nM for GnRH (185)]. This partial antagonist activity could not have been uncovered *in vivo*, thus explaining the apparent discrepancies between these results and those of Seprodi *et al.* (80). In conclusion, Rivier *et al.* (185) proposed that GnRH interacts with its receptor in a folded conformation that can be locked in by covalent bonding of the C and N termini. Most GnRH antagonists have been characterized by the lack of L-His^2 or by the substitution of L-His^2 by a D-amino acid, which resulted in selectively impairing the transducing ability of the peptide. These cyclic analogs demonstrate that at least partial agonism can also be achieved through the introduction of conformational constraints.

Based upon the fact that GnRH antagonists with drastic changes in positions 1, 2, and 3 still exhibited high binding affinity for the GnRH receptor, a variety of cyclic antagonists, particularly D- and L-Cys^1,Cys^{10} residues, were examined by Rivier *et al.* (22). A number of Cys-containing antagonists exhibited *in vitro* potencies 3–20 times lower than [D-<Glu^1,D-Phe^2,D-Trp^{3,6}]GnRH, but had no antiovulatory activity at the doses and under the conditions tested. The most potent, and interesting, however, was cyclo[-Δ^3-Pro^1,4-Cl-D-Phe^2,D-Trp^{3,6},*N*-Me-Leu^7,β-Ala^{10}-]GnRH with an *in vitro* potency equivalent to that exhibited by [D-<Glu^1,D-Phe^2,D-Trp^{3,6}] GnRH. Struthers *et al.* (186, 187) compared this constrained cyclic antagonist with GnRH (using the theoretical techniques of molecular dynamics, valence force field energy minimization, and template forcing) and derived a computer simulation of their conformation, energetics, and dynamics. It was concluded that since the cyclic antagonist was extremely rigid and had essentially a single backbone conformation (but with differing side chain conformations), this particular backbone conformation must be responsible for the ability of the antagonist to bind to the receptor. Template forcing was then used to force GnRH along a minimum energy path into the cyclic antagonist conformation. At a moderate loss of strain energy, it was found that residues 4–9 of GnRH could adopt the conformation exhibited by residues 4–9 of the cyclic antagonist. Up to this point the best cyclic antagonist showed complete antiovulatory activity in the milligram range. However, when the information derived from the molecular dynamics studies and previously reported data on Cys$^{4-9}$ and Cys$^{4-10}$ GnRH agonists (22) were combined, several 4–10 cyclic antagonists were synthesized. One of those, *e.g.* [Ac-2-D-Nal^1,4-Cl-D-Phe^2,3-D-Pal^3,Dpr^4,Arg^5,3-D-Pal^6,Asp^{10}] GnRH, with its side chains optimized for water solubility, was the most potent cyclic antagonist yet reported (185). This analog, and the corresponding Cys$^{4-10}$ derivative, showed complete inhibition of ovulation at 10–25 μg/rat using a saline vehicle and K_d values of 4.5 and 0.85, respectively. These results further support the conclusion that GnRH does, indeed, interact with its receptor in a folded conformation that can be locked in by covalent bonding of the C to N termini or through side chains of amino acids in positions 4 and 10. The results of two-dimensional proton NMR spectroscopy studies (188) on several cyclic antagonists support the proposed conformation resulting from the molecular dynamics study. A smaller cyclic hexapeptide, cyclo(-Tyr-D-Trp-Leu-Arg-Trp-Pro-), was designed by Freidinger *et al.* (189) as an antagonist based on conformational considerations (*i.e.* the suggestion of the presence of a Tyr-Gly-Leu-Arg modified Type II β-turn in GnRH), the fact that the N-terminal region which precedes the β-turn is largely hydrophobic in the most potent antagonist, and the fact that enhancement of potency is observed with hydrophobic D-amino acids in place of Gly^6. This small cyclic peptide exhibited weak *in vitro* antagonist potency approximately 3 times greater than that of the early linear antagonist, [D-Phe^2]GnRH.

The use of the γ-lactam as a conformational constraint, which was successful with GnRH (78), resulted in high *in vivo* potency when applied to an antagonist. [Ac-2-D-Nal^1,α-Me-4-Cl-D-Phe^2,D-Trp^3,2-(3′-amino-2′-oxo-1′-pyrrolidino)-4-methyl-pentanoic acid^{6,7},D-Ala^{10}] GnRH completely inhibited ovulation at 10 μg (corn oil vehicle), demonstrating, again, that a β-bend at the 6,7-position of GnRH analogs is compatible with high potency (Roeske, R., N. Chaturvedi, T. Hrinyo, and K. Kopple, unpublished observations).

Optimization of antagonist potency

The trend toward optimization of GnRH antagonist antiovulatory potency with increasing substitution is noted in Table 1. The analogs represented therein are those exhibiting the maximum potency for the given number of amino acid substitutions. While it appears that there is a relationship between increasing substitution and increased antiovulatory potency, the relation-

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TABLE 1. Maximum antiovulatory potencies of GnRH antagonists for a given number of amino acid substitutions

1 2 3 4 5 6 7 8 9 10 <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 GnRH antagonists		Total Antiovulatory dose (μg) ED_{100}
No. of substitutions	Positions(s) of substitution(s)	
1	2	—[a]
2	2, 6	6000[b]
3	2, 3, 6	750[c]
4	1, 2, 3, 6	1[d]
5	1, 2, 3, 6, 10	0.5[e]
6	1, 2, 3, 6, 7, 10	0.5[f]

[a] [D-Phe^2]GnRH (39).
[b] [4-F-D-Phe^2,D-Ala^6]GnRH (110).
[c] [D-Phe^2,Pro^3,D-Trp^6]GnRH (139).
[d] [Ac-2-D-Nal^1,4-F-D-Phe^2,D-Trp^3,D-Arg^6]GnRH (27).
[e] [Ac-2-D-Nal^1,α-Me-4-Cl-D-Phe^2,D-Trp^3,D-Arg^6,D-Ala^{10}]GnRH (Roeske, R., N. Chaturvedi, and T. Hrinyo, unpublished observations).
[f] [Ac-2-D-Nal^1,4-Cl-D-Phe^2,3-D-Pal^3,D-Arg^6,Trp^7,D-Ala^{10}]GnRH (179).

ship is, in fact, more complex. Merely increasing the number of amino acid substitutions does not necessarily lead to higher potency. The antagonists require precise topological features for high binding affinity to the receptor. The simple inversion of the D- and L-amino acid residues of a potent antagonist resulted in an analog [Ac-4-Cl-Phe^1,4-Cl-Phe^2,Trp^3,D-Ser^4,D-Tyr^5,Arg^6,D-Tyr^7,D-Arg^8,D-Pro^9,Ala^{10}]GnRH which had a relative binding affinity approximately 3000 times lower than that of its apparent mirror image [Ac-4-Cl-D-Phe^1,4-Cl-D-Phe^2,D-Trp^3,D-Arg^6,D-Ala^{10}]GnRH (Coy, D. H., M. V. Nekola, and M. J. Karten, unpublished observations). Folkers *et al.* (190), using the results of empirical energy calculations, synthesized an analog containing six D-amino acid substitutions: [Ac-Thr^1,D-Phe^2,D-Trp^3,D-Ser^4,D-Tyr^5,D-Trp^6,D-Arg^8]GnRH. Compared with the model tetrasubstituted analog [Ac-Thr^1,D-Phe^2,D-Trp^{3,6}]GnRH, it appeared to be equipotent *in vitro* and somewhat more potent *in vivo* with partial inhibition of ovulation at 25 μg, but, nevertheless, considerably less potent than the most potent tetrasubstituted analogs. Moreover, very potent superagonists with high binding affinity have been designed with only one or two substitutions, and these superagonists have K_d values equal to those of the superantagonists. Thus, the relationship between increased biological (*in vivo*) potency of antagonists and increased substitution are unclear, but conformationally related receptor binding effects and pharmacokinetic factors must be considered.

Peptide-induced histamine release

This completes the discussion of the development of GnRH antagonists with regard to attempts to design ever more potent analogs. Unfortunately, with the introduction of D-Arg and other basic side chains into position 6, came the unexpected finding by Schmidt *et al.* that a potent antagonist [Ac-2-D-Nal^1,4-F-D-Phe^2,D-Trp^3,D-Arg^6]GnRH produced transient edema of the face and extremities when administered sc in rats (191) at 1.25 mg/kg or 50–100 times the effective antiovulatory dose. This edematogenic effect was attributed to a change in vascular permeability, but it was not observed sc in mice, rabbits, or monkeys at the doses tested. Additionally, this analog was subsequently (192) shown to induce a cutaneous anaphylactoid-like reaction in rats, causing a dose-related whealing response at 10 μg/rat. At the same dose, 1.25 mg/kg, neither [Ac-Δ^3-Pro^1,4-F-D-Phe^2,2-D-Nal^{3,6}]GnRH nor the corresponding D-Trp^{3,6} analog, exhibited the edema effect (191). Similarly, a potent agonist, [N^r-Bzl-D-His^6,Pro^9-NEt]GnRH, did not exhibit this effect nor did it block the edema effects of the D-Arg^6 antagonist (191). Other potent D-Arg^6 (or D-Lys^6) antagonists showed similar edema effects (Naqvi, R. and M. Lindberg, unpublished observations). In a structure-function study of histamine release (from rat mast cells) triggered by GnRH analogs, Hook *et al.* (193) reported that [Ac-2-D-Nal^1,4-F-D-Phe^2,D-Trp^3,D-Arg^6]GnRH was 3000 times more potent than GnRH, while [D-Trp^6,Pro^9-NEt]GnRH was only 10 times more potent than GnRH, in triggering the release of histamine. By themselves, two basic side chains, in close proximity, are insufficient to impart high histamine-releasing activity to GnRH analogs. This is demonstrated by the fact that [D-Arg^6]GnRH and [Arg^6]GnRH are approximately 200 times less potent than [Ac-2-D-Nal^1,4-F-D-Phe^2,D-Trp^3,D-Arg^6]GnRH in triggering the release of histamine (Hook, W., M. J. Karten, and R. Siraganian, unpublished observations). The most potent analogs in triggering histamine release had a structural combination of a basic D-amino acid side chain at position 6 (in close proximity to the Arg^8) and a cluster of hydrophobic aromatic amino acids at the N terminus. This observation was confirmed for [Ac-4-Cl-D-Phe^{1,2},D-Trp^3,D-Arg^6,D-Ala^{10}]GnRH and for the corresponding Ac-2-D-Nal^1 analog; it was also observed for the corresponding Phe^7 modifications (194). These peptides also induced skin lesions similar to those caused by the histamine releaser, compound 48/80 (195), when tested in a cutaneous anaphylaxis test (194). Qualitatively consistent with the observation that [Ac-Δ^3-Pro^1,4-F-D-Phe^2,D-Trp^{3,6}]GnRH did not exhibit the edema effect at 1.25 mg/kg (191) or at 5 mg/kg (Naqvi, R., and M. Lindberg, unpublished observations) was the observation that this antagonist was 230 times less potent than the corresponding Ac-2-D-Nal^1-D-Arg^6 antagonist, in triggering the release of histamine (193). The transposition antagonist, [Ac-2-D-Nal^1,α-Me-4-Cl-D-Phe^2,D-Trp^3,Arg^5,D-Tyr^6,D-Ala^{10}]GnRH (182), was ap-

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TABLE 2. Nonhuman primate and/or clinical studies with GnRH antagonists

GnRH analog	References
[D-Phe^2,Phe^3,D-Phe^6]	(200)
[D-Phe^2,D-Trp^3,D-Phe^6]	(201, 202[a], 203[a], 204[a])
[D-Phe^2,Pro^3,D-Phe^6]	(25, 205)
[D-<Glu^1,D-Phe^2,D-Trp^{3,6}]	(25, 206[a])
[D-<Glu^1,D-Phe^2,D-Trp^3,D-Lys^6](N^{ϵ}-isophthaloyl-Lys^6 dimer)	(207[a])
Endo-Pro^{1a}[D-Phe^2,D-Trp^{3,6}]	(25, 205)
[Ac-Pro^1,D-Phe^2,D-Trp^{3,6}]	(25)
[Ac-Δ^3-Pro^1,4-Cl-D-Phe^2,D-Trp^{3,6}]	(208)
[Ac-Δ^3-Pro^1,4-F-D-Phe^2,D-Trp^{3,6}]	(209, 210, 211[a])
[Ac-Δ^3-Pro^1,4-Cl-D-Phe^2,2-D-Nal^{3,6}]	(212)
[Ac-Δ^3-Pro^1,4-F-D-Phe^2,2-D-Nal^{3,6}]	(119)
[Ac-Δ^3-Pro^1,4-Cl-D-Phe^2,D-Trp^{3,6},N-Me-Leu^7]	(208)
[Ac-D-Phe^1,4-Cl-D-Phe^2,D-Trp^{3,6}]	(213[a])
[Ac-D-Trp^1,4-Cl-D-Phe^2,D-Trp^3,D-Phe^6,D-Ala^{10}]	(214)
[Ac-4-Cl-D-Phe^{1,2},D-Trp^3,D-Phe^6,D-Ala^{10}]	(215[a])
[Ac-4-Cl-D-Phe^{1,2},D-Trp^3,D-Arg^6,D-Ala^{10}]	(216–221)
[Ac-2-D-Nal^1,4-F-D-Phe^2,D-Trp^3,D-Arg^6]	(222), ([b])[a]
[Ac-2-D-Nal^1,4-Cl-D-Phe^2,D-Trp^3,N^{ω},$N^{\omega'}$-diethyl-D-Har^6,D-Ala^{10}]	(223–225), ([c])[a]

[a] Denotes references to clinical studies.
[b] Crowley, W. F., unpublished results.
[c] Henzl, M., unpublished results.

proximately 20–40 times less potent than the D-Arg^6 antagonists in triggering histamine release; hence separation of the two arginine residues partially reduces the histamine release potential of these antagonists. The diethyl-homoarginine^6 analog, [Ac-2-D-Nal^1,4-F-D-Phe^2,D-Trp^3,N^{ω},$N^{\omega'}$-diethyl-Har^6,D-Ala^{10}]GnRH was half as potent as [Ac-2-D-Nal^1,4-F-D-Phe^2,D-Trp^3,D-Arg^6]GnRH in triggering the release of histamine (Hook, W., M. J. Karten, and R. Siraganian, unpublished observations). Thus, it appears that GnRH agonists and antagonists, as well as a variety of other peptides such as substance P, somatostatin, vasoactive intestinal peptide, gastrin, *etc.* (195–199), trigger the release of histamine, apparently as a function of certain structural parameters that are independent of other inherent biological activities. Current structural modifications of GnRH antagonists are being undertaken to drastically reduce the histamine release potential while maintaining and/or increasing the GnRH antagonist potency.

Clinical Explorations

The relatively few completed and current clinical trials as well as the more extensive nonhuman primate studies have been cited in Table 2. The earliest trisubstituted GnRH antagonists to be tested in nonhuman primates or humans contained D-Phe in positions 2 and 6 and an L- or a D-amino acid in position 3. The next generation of antagonists to be tested in primates, the tetrasubstituted analogs, all contained hydrophobic D-amino acids in positions 2, 3, and 6 (D-Phe^2 or 4-Cl (or F)-D-Phe^2 and D-Trp^{3,6} or 2-D-Nal^{3,6}) and an L- or a D-amino acid in position 1. The current generation of tetra- and pentasubstituted antagonists being explored clinically, or in nonhuman primates, all contain hydrophobic D-amino acids in positions 1, 2, and 3 [Ac-4-Cl-D-Phe^1 or Ac-2-D-Nal^1,4-Cl(or F)-D-Phe^2 and D-Trp^3] and a basic D-amino acid in position 6 (D-Arg^6 or N^{ω},$N^{\omega'}$-diethyl-Har^6). The pentasubstituted analogs also contain D-Ala in position 10.

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