In Vitro and *in Vivo* Activities of Reduced-Size Antagonists of Luteinizing Hormone-Releasing Hormone[†,§]

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A novel series of octapeptide LHRH antagonists was designed on the basis of the structure of the (2–9) fragment of a LHRH agonist. By adopting a systematic SAR study, we were able to improve first the *in vitro* activity and then the *in vivo* LH suppression, raising them up to the range of the decapeptide antagonists NalGlu (**51**) and A-75998 (**50**), resulting in A-76154 (**49**). The octapeptide antagonist A-76154 is the most potent reduced-size LHRH antagonist reported. It suppresses LH in the castrated rat by over 80% for a period of 4 h following sc bolus administration of 30 μg/kg.

Introduction

Antagonists of luteinizing hormone-releasing hormone (LHRH, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH$_2$) bind to the LHRH receptor in the pituitary gonadotrophs causing inhibition of gonadotropin release, which subsequently causes the suppression of sex steroids in mammals.[1,2] This property of suppressing sex hormones renders the LHRH antagonists potentially useful in the treatment of a large variety of endocrine-based conditions such as prostate cancer, endometriosis, and precocious puberty.[1,2] Several LHRH agonists are currently used in the treatment of the above indications.[3,4] Because of their peculiar mechanism of action, the LHRH agonists suppress gonadotropins and sex hormones only following chronic administration for 2–4 weeks. In the first days of administration agonists increase plasma gonadotropins and sex steroids, occasionally causing an initial clinical flare.[1,2] Antagonists, on the other hand, are devoid of this initial hormone surge.

Over the last 10 years a large number of LHRH antagonists have been synthesized and tested.[5] All of them are either nona- or decapeptides containing four or five D-amino acids. We previously reported a series of reduced-size hexapeptide analogues of LHRH, both agonists and antagonists,[6] which contain only one D-amino acid and were designed based on the (3–9) fragment of the agonist [DLeu^6,Pro^{9}NHEt]LHRH, known as leuprolide. In that series, the most potent antagonist, [*N*-(1-naphthylpropionyl)-Ser^4,DLeu^6,Pro^{9}NHEt](3–9)LHRH, had a pK_I of 9.55 for receptor binding and a pA_2 of 9.28 for *in vitro* LH inhibition. However, *in vivo* in the castrated rat model the compound was active only by infusion, presumably because of a short duration of action.[6] In an attempt to increase both the *in vitro* potency and the *in vivo* duration of action, we decided to evaluate other fragments of LHRH analogues as the departure point for the design of a novel series of reduced-size LHRH antagonists, which would be active in the range of standard decapeptide antagonists following sc bolus injection in the rat. As our starting point we selected the longer (2–9) fragment (**2**, Table 1) of the agonist [Phe^2,DTrp^6,Pro^{9}NHEt]LHRH (**1**),[8] which had a pK_I of 7.08 and a pD_2 of 7.64 (for definitions of pK_I and pD_2, see footnotes of Table 1). The compounds were tested *in vitro* for LHRH rat pituitary receptor binding, for suppression of LH release from rat pituitary cells and release of histamine from rat peritoneal mast cells, and *in vivo* for LH suppression in the castrated rat.

Chemical Synthesis

All the peptides were synthesized by solid-phase synthesis techniques (SPPS).[7] Peptides containing Pro-ethyl amide at the C-terminus were synthesized using Boc-Pro attached to Merrifield resin, and the peptide was cleaved from the resin with ethylamine. Peptides containing DAla amide at the C-terminus were synthesized using Boc-DAla attached to 4-methylbenzhydrylamine resin, and the peptide was cleaved with anhydrous HF.[8-10] The synthesis protocol, cleavage of the peptide from the resin, removal of the protecting groups, workup, and HPLC purification were analogous to those described in our recent publications.[8,9] All the peptides were characterized by analytical HPLC, FABMS, and AAA.

Biological Testing

Antagonists were tested *in vitro* for LHRH receptor binding[6] and for LH release from cultured rat pituitary cells.[6] The binding affinities are reported as pK_I. The *in vitro* LH release activities of agonists are reported as pD_2. The LH inhibition potencies for antagonists are reported as pA_2 (for definition of pA_2, see footnote of Table 1). For initial characterization of the safety profile, the antagonists were tested for histamine release from rat peritoneal mast cells.[11,12]

All antagonists were tested for LH suppression in castrated male rats following sc administration of 30 μg/kg dose, dissolved in 1:4 propylene glycol/saline solution, to groups of three animals. Serial blood samples were collected for 24 h after dosing. Plasmas were separated by centrifugation and frozen at −20 °C until assayed. LH

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[§] Abbreviations: The abbreviations for the amino acids are in accordance with the recommendations of the IUPAC–IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.* 1984, *138*, 9–37). The symbols represent the L-isomer except when indicated otherwise. Additional abbreviations: DlNal, D-3-(1-naphthyl)alanine; NMeTyr, *N*-methyltyrosine; DLys(Nic), D-lysine(*N*-ε-nicotinyl); Lys(Isp), lysine-(*N*-ε-isopropyl); DGlu(AA), 4-(*p*-methoxybenzoyl)-D-2-aminobutyric acid; HPLC, high-pressure liquid chromatography; LH, luteinizing hormone; sc, subcutaneous; FABMS, fast atom bombardment mass spectrum; AAA, amino acid analysis.

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Table 1. *In Vitro* Functional Properties of Reduced-Size LHRH Antagonists

H[1]-Phe[2]-Trp[3]-Ser[4]-Tyr[5]-DTrp[6]-Leu[7]-Arg[8]-Pro[9]-NHEt

compd	substitution	t_R[a]	MH$^+$ [b]	pK_I[c]	pD_2[d]	pA_2[e]	ED$_{50}$[f]
1	pGlu[1] [g]			10.61 (±0.05)	10.81 (±0.13)		
2	as above	15.83	1181	7.08 (±0.28)	7.64 (±0.33)		
3	NAcPhe[2]	20.00	1223	9.78 (±0.13)		8.02 (±0.16)	
4	phenylacetyl[2]	44.46	1152	9.08 (±0.33)		8.26 (±0.26)	
5	(4-chlorophenyl)acetyl[2]	47.15	1186	9.08 (±0.33)		8.26 (±0.26)	
6	(4-hydroxyphenyl)acetyl[2]	40.03	1168	9.09 (±0.28)		7.35 (±0.31)	
7	indolyl-2-carbonyl[2]	37.12	1177	7.77 (±0.13)		7.75 (±0.225)	
8	(4-chlorophenyl)propionyl[2]	49.50	1200	10.28 (±0.1)		9.90 (±0.4)	
9	(4-fluorophenyl)propionyl[2]	43.87	1184	10.48 (±0.14)		9.23 (±0.27)	
10	(4-methoxyphenyl)propionyl[2]	46.01	1196	10.11 (±0.19)		8.62 (±0.22)	
11	[4-(trifluoromethyl)phenyl]propionyl[2]	45.65	1234	9.56 (±0.14)		9.02 (±0.30)	
12	(2-fluorophenyl)propionyl[2]	44.89	1184	10.24 (±0.05)		9.35 (±0.09)	
13	(4-hydroxyphenyl)propionyl[2]	39.67	1182	10.07 (±0.23)		7.81 (±0.28)	
14	(3-fluorophenyl)propionyl[2]	46.29	1184	8.87 (±0.16)		9.05 (±0.05)	
15	[4-(trifluoromethyl)phenyl]acetyl[2],DTrp[3]	35.66	1220	9.19 (±0.12)		8.58 (±0.09)	
16	indolyl-2-carbonyl[2],DTrp[3]	44.29	1205	10.12 (±0.23)		9.07 (±0.05)	
17	5-fluoroindolyl-2-carbonyl[2],DTrp[3]	47.20	1195	7.91 (±0.15)		7.38 (±0.04)	
18	(4-fluorophenyl)propionyl[2],DTrp[3]	47.14	1184	10.54 (±0.01)		10.41 (±0.31)	
19	(4-chlorophenyl)propionyl[2],DTrp[3]	44.60	1200	10.12 (±0.14)		9.78 (±0.06)	
20	(4-chlorophenyl)propionyl[2],DTyr[3]	44.16	1177	10.75 (±0.25)		10.08 (±0.125)	
21	(4-chlorophenyl)propionyl[2],Tyr[3]	40.18	1177	10.12 (±0.23)		9.07 (±0.05)	
22	(2,4-difluorophenyl)propionyl[2],DTrp[3]	46.75	1202	10.40 (±0.08)		10.85 (±0.05)	
23	(3,4-difluorophenyl)propionyl[2],DTrp[3]	47.51	1200	10.28 (±0.27)		10.45 (±0.05)	
24	(4-fluorophenyl)propionyl[2],D4ClPhe[3]	49.80	1179	10.05 (±0.0)		10.75 (±0.15)	
25	(4-fluorophenyl)propionyl[2],DTyr[3]	41.41	1161	10.65 (±0.06)		9.66 (±0.13)	
26	(4-fluorophenyl)propionyl[2],D1Nal[3]	50.23	1195	10.77 (±0.18)		11.15 (±0.15)	
27	(4-fluorophenyl)propionyl[2],1Nal[3]	51.24	1195	10.77 (±0.17)		11.30 (±0.0)	
28	(4-fluorophenyl)propionyl[2],DPro[3]	41.89	1095	10.93 (±0.21)		8.62 (±0.29)	
29	(4-fluorophenyl)propionyl[2],DLys(Nic)[6]	37.21	1231	9.46 (±0.22)		7.68 (±0.25)	
30	(4-fluorophenyl)propionyl[2],DTrp[3],DLys(Nic)[6]	35.88	1231	10.63 (±0.03)		10.02 (±0.28)	
31	(4-fluorophenyl)propionyl[2],DTyr[6]	41.34	1161	10.42 (±0.24)		7.90 (±0.24)	
32	(4-fluorophenyl)propionyl[2],D4ClPhe[6]	50.04	1179	10.74 (±0.21)		11.05 (±0.25)	
33	(4-fluorophenyl)propionyl[2],DTrp[3],DTyr[6]	41.24	1161	10.99 (±0.0)		10.50 (±0.10)	
34	(4-fluorophenyl)propionyl[2],DTrp[3],NMSer[4]	46.35	1199	9.87 (±0.15)		9.94 (±0.02)	
35	(4-fluorophenyl)propionyl[2],DTrp[3],Pro[4]	49.89	1194	9.08 (±0.18)		7.67 (±0.01)	
36	(4-fluorophenyl)propionyl[2],DTrp[3],Pro(4OH)[4]	46.55	1210	8.89 (±0.0)		7.68 (±0.0)	
37	(4-fluorophenyl)propionyl[2],DTrp[3],NMeTyr[5]	35.79	1198	10.48 (±0.08)		11.15 (±0.05)	
38	(4-fluorophenyl)propionyl[2],DTrp[3],NMeTyr[5],DLys(Nic)[6]	34.63	1245	10.78 (±0.24)		10.46 (±0.10)	<0.1

Table 1. (Continued)

compd	substitution	t_R[a]	MH$^+$ [b]	pK_I[c]	pD_2[d]	pA_2[e]	ED$_{50}$[f]
39	(2,4-difluorophenyl)propionyl^2,DTrp^3,NMeTyr^5,DLys(Nic)^6	36.10	1263	10.69 (±0.25)		11.05 (±0.35)	
40	(3-fluorophenyl)propionyl^2,DTrp^3,NMeTyr^5,DLys(Nic)^6	35.38	1245	10.58 (±0.04)		10.06 (±0.19)	
41	(4-chlorophenyl)propionyl^2,DTrp^3,NMeTyr^5,DLys(Nic)^6	37.50	1261	10.69 (±0.19)		10.80 (±0.0)	
42	(2,4-difluorophenyl)propionyl^2,DTrp^3,NMeTyr^5	47.73	1216	10.41 (±0.09)		11.00 (±0.10)	
43	(4-chlorophenyl)propionyl^2,DTrp^3,NMeTyr^5	49.34	1214	10.15 (±0.32)		11.40 (±0.10)	
44	(4-fluorophenyl)propionyl^2,D1Nal^3,NMeTyr^5	50.88	1209	10.32 (±0.38)		11.25 (±0.15)	
45	(4-bromophenyl)propionyl^2,D1Nal^3,NMeTyr^5	54.23	1271	10.25 (±0.10)		11.20 (±0.30)	
46	(4-fluorophenyl)propionyl^2,D1Nal^3,NMeTyr^5,DLys(Nic)^{6}Lys(Isp)^8	41.55	1270	11.62 (±0.0)		11.43 (±0.23)	
47	(4-fluorophenyl)propionyl^2,D1Nal^3,NMeTyr^5,DLys(Nic)^6,Lys(Isp)^8,-Pro^{9}NH$_2$	20.08	1242	10.80 (±0.27)		11.16 (±0.70)	
48	(4-fluorophenyl)propionyl^2,D1Nal^3,NMeTyr^5,DLys(Nic)^6,Lys(Isp)^8,-Pro^{9}OH	20.67	1243	9.27 (±0.11)		8.11 (±0.05)	
49	(4-fluorophenyl)propionyl^2,D1Nal^3,NMeTyr^5,DLys(Nic)^6,Lys(Isp)^8,-DAla^{10}NH$_2$	38.97	1313	10.69 (±0.25)		11.13 (±0.13)	10.30 (±0.25)
50	A-75998[h]			10.50 (±0.20)		11.23 (±0.14)	10.00 (±3.6)
51	NalGlu[i]			10.28 (±0.22)		11.06 (±0.20)	1.11 (±0.12)

[a] Analytical HPLC retention time in minutes. HPLC conditions are described in the Experimental Section. [b] Values determined by FABMS. [c] The negative logarithm of the concentration of analogue that inhibits 50% of the binding of ^{125}I-labeled leuprolide to the rat pituitary LHRH receptor. [d] The negative logarithm of the concentration of agonist that produces 50% of the maximum release of LH from cultured rat pituitary cells. [e] The negative logarithm of the concentration of antagonist that requires 2-fold higher concentration of agonist to release LH from cultured rat pituitary cells. [f] Effective dose of antagonist that gives 50% of maximal release of histamine from rat peritoneal mast cells. Units are micrograms per milliliter. [g] Reference 8. [h] Reference 9. [i] Reference 13.

levels were measured by radioimmunoassay.[8] All the compounds were evaluated as the TFA salts.

Results and Discussion

Elimination of the N-terminal residue pGlu^1 from agonist [Phe^2,DTrp^6,Pro^{9}NHEt]LHRH (**1**, Table 1) caused over 1000-fold loss in receptor binding affinity (**2**), but still maintained a weak agonistic activity with pD_2 of 7.64. Acylation of the N-terminus of peptide **2** to give **3** increased the receptor binding affinity by 500-fold (pK_I 9.78) and converted the compound to antagonist (pA_2 8.02). This interesting result was similar to our previous observation in the hexapeptide reduced-size LHRH analogues where we found that the size, shape, and conformation of the substituent at positions 3 and 6 could influence the response for either agonist or antagonist.[6] It is also in agreement with our previous observation that substitution of NMePhe^2 in leuprolide converted the agonist to antagonist.[8] It is quite astonishing how such a small structural change in these LHRH analogues can cause an agonist/antagonist switch.

It is also worth noting the large difference between the pK_I and pA_2 values of compound **3** (9.78 and 8.02, respectively). We do not have an explanation for this discrepancy which, as is shown later, disappears with increase in potency. Substitution of analogue **2** with phenylacetyl^2 reduced the binding affinity 5-fold (**4**). Elongation of the residue 2 side chain to propionyl and substitution of the benzene ring with 4-Cl or 4-F increased the pK_I to 10.28 and 10.48 and raised the pA$_2$ to 9.90 and 9.23, respectively (**8** and **9**).[5] The 4-(chlorophenyl)-propionyl residue mimics the D4ClPhe^2 typically found at this position in LHRH antagonists.[5] Compounds **8** and **9**, when tested *in vivo* in the castrated rat at 30 μg/kg sc bolus, were inactive. In an attempt to increase metabolic stability, we substituted DTrp^3 in analogues **7**, **8**, **9**, and **11**, generating antagonists **16**, **19**, **18**, and **15**, respectively. The new analogue, [(4-fluorophenyl)propionyl^2, DTrp^{3,6},Pro^{9}NHEt](3–9)LHRH (**18**), had approximately equal pK_I and pA_2 values and was active *in vivo* following bolus sc administration. In the castrated rat antagonist **18** suppressed LH by 88% after 2 h (Figure 1A). To increase hydrophobicity we substituted D1Nal^3 in **18**, yielding antagonist **26** which, for the first time in this series, had a pA_2 higher than its pK_I, and both values were in the range of the decapeptide antagonists NalGlu, NAcD2Nal-D4ClPhe-D3Pal-Ser-Arg-DGlu(AA)-Leu-Arg-Pro-DAlaNH$_2$ (**51**),[13] and A-75998, NAc-D2Nal-D4ClPhe-D3Pal-Ser-NMeTyr-DLys(Nic)-Leu-Lys(Isp)-Pro-DAlaNH$_2$ (**50**),[9] which currently is undergoing clinical studies. However, compound **26** was also inactive in the castrated rat at the standard dose. To gain *in vivo* activity we substituted a large variety of groups at positions 3, 4 and 6 (**20-36**, Table 1). Although several of the new compounds (**23**, **24**, **27**, **32**, and **33**) had pA_2 values above 10.50, they were still inactive *in vivo*, except for compound **30**, which suppressed LH in the castrated rat by 70% for a period of less than 1 h (Figure 1A).

To further increase metabolic stability and improve pharmacokinetics we substituted NMeTyr^5 in compound **18** producing analogue **37**, which *in vivo* suppressed LH by 79% and maintained suppression for 4 h (Figure 1B). To increase hydrophilicity and improve the pharmacokinetic profile, we substituted DLys(Nic)^6 in analogue **30**. The new antagonist **38** was active *in vitro* (pK_I 10.78, pA_2 10.46) and in the castrated rat caused 90% testosterone suppression 2 h after dosing (Figure 1B).

Having improved the *in vitro* and *in vivo* activities of our compounds, we began to evaluate them for histamine

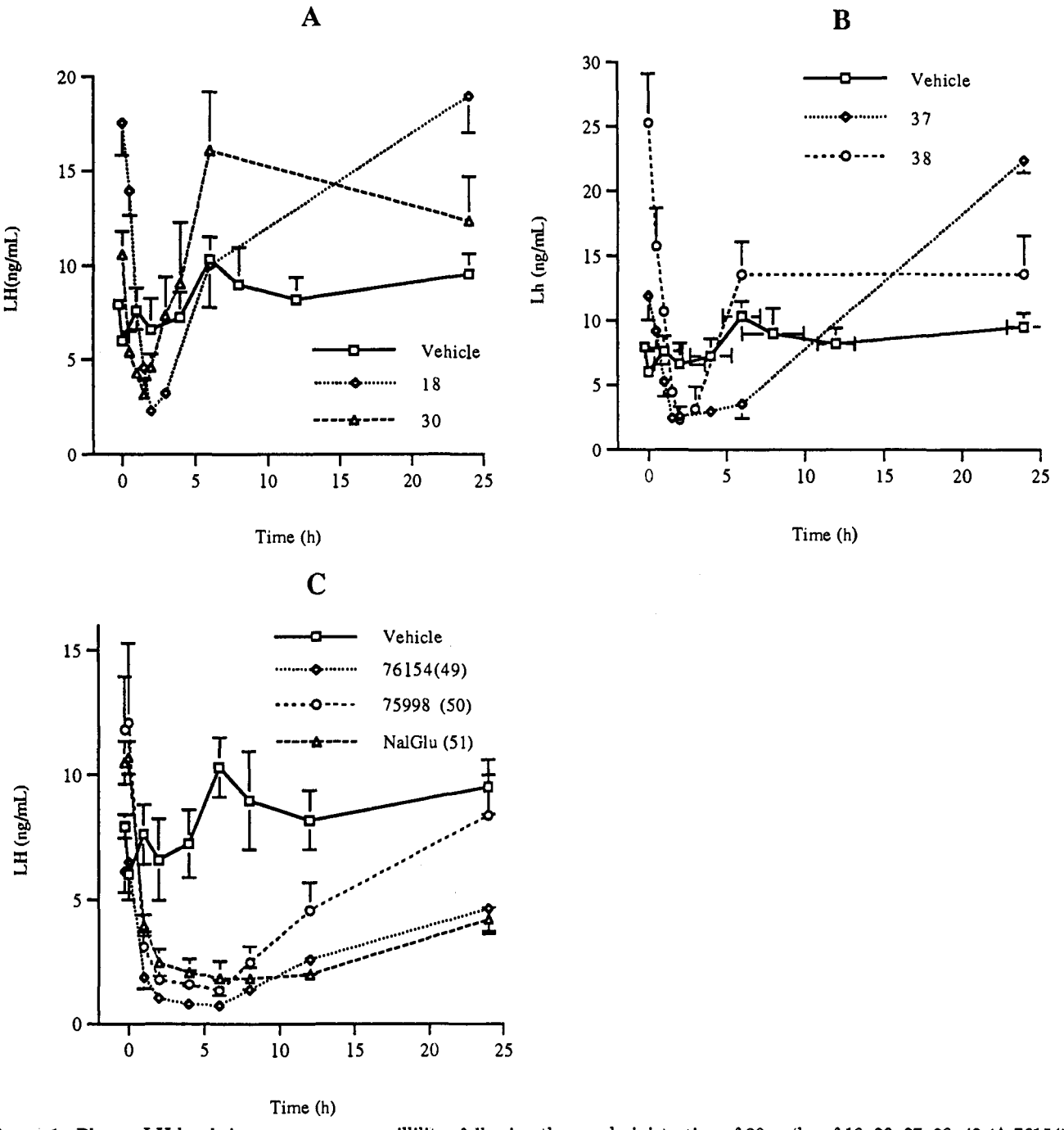

Figure 1. Plasma LH levels in nanograms per milliliter following the sc administration of 30 μg/kg of **18**, **30**, **37**, **38**, **49** (A-76154), **50** (A-75998), and **51** (NalGlu) antagonists to male castrated rats.

release (HR) in the rat peritoneal mast cell assay. Unfortunately, when compound **38** was tested, it had an ED_{50} that was 10-fold lower than A-75998 (Table 1), which we considered inadequate. In an effort to decrease the compound reactivity for histamine and improve pharmacokinetics, we continued modifying positions 2, 3, 8, and 10 (Table 1), finally leading to antagonist **49**, [*N*-[(4-fluorophenyl)propionyl]-D1Nal[3],NMeTyr[5], DLys(Nic)[6],Lys(Isp)[8],DAla[10]NH_2](3–10)LHRH, known as A-76154. This compound *in vitro* and *in vivo* (Table 1, Figure 1C) was as active as the standard antagonists A-75998 and NalGlu.[9,13] A-76154 had an ED_{50} for HR 10-fold higher than that of NalGlu and identical to that of A-75998.

In summary, we found that substitutions of D1Nal[3], NMeTyr[5], and DAla[10] were crucial to improve the pharmacokinetics and the *in vivo* activity, whereas substitution of Lys(Isp)[8] was essential to increase the HR ED_{50}.[14] Interestingly, residues 4–10 of the octapeptide antagonist A-76154 which was developed by a thorough SAR study of a (2–9) fragment of a LHRH agonist, are identical to those in A-75998, a decapeptide antagonist which was designed based on the structure of antide.[9]

Conclusions

We have developed the most potent reduced-size series of LHRH antagonists which were very effective in suppressing LH in the castrated rat following sc bolus administration. A-76154 is an octapeptide antagonist representative of this series, which was active in the range of NalGlu and A-75998, two decapeptide LHRH antagonists which have been also tested in clinical settings.

Experimental Section

All the peptides were synthesized using a Milligen-Biosearch Model 9500 automated peptide synthesizer (Milligen-Biosearch, Division of Millipore, Burlington, MA). The HF-reaction ap-

paratus, Type 1B, was from Peninsula Laboratories, Inc., Belmont, CA. Peptide purification was performed with a Rainin/Gilson Ternary HPLC system. FABMS were run using a Finningan MAT, MAT90 double-focusing magnetic sector (BE) mass spectrometer, xenon FAB ionization, and 1:1 glycerol/thioglycerol matrix. Amino acid analyses were performed on a Beckman Model 6300 amino acid analyzer, using Ninhydrin derivatization. The peptides were hydrolyzed with 6 N HCl containing 0.5% phenol at 150 °C for 2 h. The data handling system was PE Nelson ACCESS CHROM. For calibration, Beckman standards were used. The values for Ser and Trp were generally low because of partial decomposition. The content of Ala, Pro, Leu, Lys, and Arg were within ±10%. We did not look for the presence of any unnatural amino acid. That was confirmed by FABMS.

The following Boc-protected amino acids, Boc-Ser(OBzl), Boc-Tyr(O-2Br-Cbz), Boc-NMeTyr(O-2,6-di-Cl-Bzl), Boc-DLys(N-ε-Nicotinyl), Boc-Leu, Boc-Arg(Tos), Boc-Lys(N-ε-isoproyl-N-ε-Cbz)DCHA, Boc-Pro, were purchased from Bachem Inc. (Torrance CA). Boc-D1Nal was purchased from Synthetech Inc. (Albany, OR). TFA was obtained from Kali-Chemie Co. Inc. (Greenwich, CT). Boc-Pro Merrifield resin and Boc-DAla-4-methylbenzhydrylamine resin (with a substitution varying from 0.4 to 0.7 mmol/g) were obtained from Peninsula Laboratories, Inc., (Belmont, CA). (4-Fluorophenyl)propionic acid was obtained by catalytic hydrogenation (Pd/C) of 4-fluorocinnamic acid. All the solvents were purchased from Fisher Scientific Co. (Fair Lawn, NJ). HF gas cylinders were purchased from AGA Gas Inc., Cleveland, OH. All other chemicals were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI).

General Synthetic Method for the SPPS and Purification of Peptides 2–49. A typical synthesis for a peptide used 0.8 g of Boc-D-Merrifield resin for peptides containing ProNHEt and Boc-DAla-4-methylbenzhydrylamine resin for peptides containing $DAlaNH_2$. The synthetic protocol, the conditions for cleavage of resin and protecting groups, the workup, and HPLC purification for peptides containing ProNHEt at the C-terminus were analogous to that described for the (1-9) LHRH agonists.[8] The synthetic protocol, the conditions for cleavage of resin and protecting groups, the workup, and HPLC purification for peptides containing $DAlaNH_2$ at the C-terminus were analogous to that used for the (1–10) LHRH antagonists.[9] Analytical HPLC separation was achieved with a C_{18} Dynamax column (0.46 × 25 cm), 300-Å pore size, 5-μm particle size fitted with a guard column of the same material (0.46 × 1.5 cm). The solvent system was 0.1% TFA in water/acetonitrile and the gradient was 25%–60% acetonitrile over 35 min. The UV detector was set at 254 nm. Preparative HPLC separation was accomplished with a C_{18} Dynamax column (2.14 × 25 cm), 60-Å pore size, 8-μm particle size, with a guard column of the same material (2.14 × 5.0 cm). Eighty fractions (0.5 min each) were collected in the interval from 10 to 50 min. Each fraction was checked for purity by analytical HPLC. The pure fractions were combined and lyophilized to provide a homogeneous fluffy white powder. The purity of the final compounds was over 95% on the basis of analytical HPLC, FABMS, and AAA.

***In Vitro* Biological Assays.** All compounds were tested in the receptor binding and LH-release assays which we have previously described.[6] The histamine-release assay from rat peritoneal mast cells followed a reported procedure.[11,12]

***In Vivo* LH Inhibition.** The assay procedure was described in our recent publication.[9]

Supplementary Material Available: Amino acid analysis data for compounds 2–49 (3 pages). Ordering information is given on any current masthead page.

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