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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 15 (2005) 2894–2897

Synthesis, in vivo and in vitro biological activity of novel azaline B analogs

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Received 8 February 2005; revised 18 March 2005; accepted 21 March 2005
Available online 27 April 2005

Abstract—Several azaline B analogs (**2**–**10**) were synthesized and evaluated for their ability to antagonize GnRH in vitro and for duration of action in inhibiting luteinizing hormone secretion in a castrated male rat assay in vivo. Analogs, **8** (IC_{50} = 1.85 nM), and **9** (IC_{50} = 1.78 nM), are equipotent with azaline B (**1**, IC_{50} = 1.36 nM) in vitro. Whereas **9** is short acting, **8** is as long acting as azaline B. Other analogs have IC_{50} greater than 2.0 nM and are all short acting.

Gonadotropin-releasing hormone (GnRH) is a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) isolated and characterized[1–3] by the groups of the 1977 Nobel laureates, Schally and Guillemin. GnRH is synthesized in the hypothalamus and acts upon the receptors in the anterior pituitary where it triggers the synthesis and release of the luteinizing hormone (LH) and follicle-stimulating hormone (FSH).[4] GnRH superagonists and antagonists have been widely studied[5] for the treatment of prostate and breast tumors, uterine fibroids, precocious puberty, endometriosis, premenstrual syndrome, contraception and infertility. GnRH antagonists may have a clinical advantage over superagonists because they lower gonadal sex hormone levels almost immediately and avoid the initial upregulation of the gonadotropin-gonadal axis (flare effect) caused by the use of superagonists.[6]

Azaline B (**1**), discovered in our laboratory,[7] is one of the most potent and long-acting GnRH antagonists. Because of its limited solubility in aqueous buffers, its clinical development was abandoned generating a need for more potent antagonists with improved physicochemical properties, such as ease of formulation for acute or slow release, extended duration of action and economical simpler synthesis. All of these properties may be fulfilled by closely related analogs of azaline B. Herein we describe a series of azaline B derivatives (Ac-D-2Nal[1]-D-4Cpa[2]-D-3Pal[3]-Ser[4]-4Aph(X)[5]-D-Aph(Cbm)[6]-Leu[7]-ILys[8]-Pro[9]-D-Ala[10]-NH_2) wherein the ω-amino function of the 4-aminophenylalanine (Aph) at position 5 was acylated with N^{α}-carbamoylated amino acids, dicarbamoylated diamino acids and with different carboxylic acids bearing lactam/urea functionalities. The acylated groups (X) at position 5 were introduced to (i) increase the number of potential intra- and intermolecular hydrogen bonding sites on the peptide side chains for structural stabilization and peptide/receptor/plasma protein interactions, (ii) increase solubility in aqueous buffers, and (iii) decrease the propensity of azaline B and congeners to form gels under certain conditions.[8] The effects of these substitutions on the biological activity were evaluated in vitro and in vivo.

Azaline B (**1**) was synthesized as previously described.[7] All of the other analogs (**2**–**10**, Table 1) were derived from the resin-bound peptide precursor [Ac-D-Nal-D-Cpa-D-Pal-Ser(Bzl)-Aph(N^{ω}-Fmoc)-D-Aph(Cbm)-Leu-ILys(N^{α}-Z)-Pro-D-Ala]-MBHA resin, synthesized manually on *p*-methylbenzhydrylamine resin (1 g, 0.33 mequiv NH_2/g) using solid phase peptide synthesis (SPPS) techniques[9] and N^{α}-Boc strategy. In the synthesis of **2**–**4**, the Fmoc protection on Aph at position 5 was removed by the treatment with 30% piperidine in *N*-methylpyrrolidone

Keywords: GnRH antagonists; Structure activity relationships; Azaline B; Degarelix.
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doi:10.1016/j.bmcl.2005.03.085

Table 1. Physicochemical characterization and biological activities of GnRH antagonists

No	Compound	Purity		t_R[c] (min)	MS[d] (M + H)$^+$		pIC$_{50}$[e] Ave ± SEM	IC$_{50}$ (nM)[f]	Duration of action[g]
		HPLC[a]	CZE[b]		Calc.	Obs.			
1	[Ac-D-2Nal^1, D-4Cpa^2, D-3Pal^3, 4Aph(Atz)^5, D-4Aph(Atz)^6, ILys^8, D-Ala^{10}]GnRH (azaline B)	98	99	27.7	1612.8	1612.7	8.9 ± 0.07	1.36	Long
2	[Aph(N^{α}-Cbm-Asn)^5, D-Aph(Cbm)^6]azaline B	95	98	26.7	1648.8	1648.8	8.7 ± 0.17	2.22	Short
3	[Aph(N^{α}-Cbm-DAsn)^5, D-Aph(Cbm)^6]azaline B	96	95	26.8	1648.8	1648.8	8.6 ± 0.10	2.37	Short
4	[Aph(N^{α}-Cbm-Gln)^5, D-Aph(Cbm)^6]azaline B	98	96	26.6	1662.8	1662.8	8.2 ± 0.02	6.86	Short
5	[Aph(N^{α},N^{β}-di-(Cbm)Dap)^5, D-Aph(Cbm)^6]azaline B	95	95	27.2	1663.8	1663.8	8.2 ± 0.01	6.37	Short
6	[Aph(N^{α},N^{β}-di-(Cbm)D-Dap)^5, D-Aph(Cbm)^6]azaline B	96	97	27.3	1663.8	1663.7	8.5 ± 0.19	2.82	Short
7	[Aph(N^{α},N^{γ}-di-(Cbm)Dab)^5, D-Aph(Cbm)^6]azaline B	95	95	27.1	1677.8	1677.8	8.5 ± 0.08	3.53	Short
8	[Aph(L-pGlu)^5, D-Aph(Cbm)^6]azaline B	99	97	28.1	1602.8	1602.8	8.7 ± 0.11	1.85	Long
9	[Aph((L-tetrahydro-pyrimidine-2-one)-6-carboxyl)^5, D-Aph(Cbm)^6]azaline B	95	98	28.3	1617.8	1617.8	8.7 ± 0.00	1.78	Short
10	[Aph(thymine-1-acetyl)^5, D-Aph(Cbm)^6]azaline B	99	98	30.2	1657.8	1657.7	8.6 ± 0.5	2.46	Short

Abbreviations: Ac, acetyl; Aph, 4-aminophenylalanine; Atz, [5′-(3′-amino-1*H*-1′,2′,4′-triazolyl)]; Cbm, carbamoyl; 4Cpa, 4-chlorophenylalanine; Dab, α,γ-diaminobutyric acid; Dap, α,β-diaminopropionic acid; ILys, N^{ε}-isopropyllysine; 2Nal, 3-(2-naphthyl)alanine; 3Pal, 3-(3-pyridyl)alanine, pGlu, pyroglutamic.

[a] Percentage purity determined by HPLC using buffer system A; TEAP, pH 2.30, buffer system B, 60% CH_3CN/40% A under gradient conditions (30–80% B over 50 min), at flow rate of 0.2 mL/min on a Vydac C_{18} column (0.21 × 15 cm, 5 μm particle size, 300 Å pore size). Detection at 214 nm.

[b] Percentage purity determined by capillary zone electrophoresis (CZE) using a Beckman P/ACE System 2050 controlled by an IBM Personal system/2 model 50Z; field strength of 15 kV at 30 °C. Buffer, 100 mM sodium phosphate (85:15, H_2O:CH_3CN), pH 2.50, on a Agilent μSil bare fused-silica capillary (75 μm i.d. × 40 cm length). Detection at 214 nm.

[c] Retention times under gradient conditions (30–80% B over 50 min); buffer system A; TEAP, pH 7.0, buffer system B, 60% CH_3CN/40% A.

[d] Mass spectra (MALDI-MS) were measured on an ABI-Voyager DESTR instrument using saturated solution of α-cyano-4-hydroxycinnamic acid in 0.3% trifluoroacetic acid and 50% acetonitrile as matrix. The calculated $[M+H]^+$ of the monoisotope compared with the observed $[M+H]^+$ monoisotopic mass.

[e] The pIC$_{50}$ is the negative log of the IC$_{50}$ in molar, as determined in the GnRH reporter gene assay.

[f] IC$_{50}$ is the concentration of antagonist required to repress the GnRH induced response by 50% in the reporter gene assay in HEK-293 cells expressing the human GnRH receptor and a GnRH-responsive stably integrated luciferase reporter gene. The values shown represent the geometric mean from two independent experiments.

[g] Castrated male rat assay. Duration of action: long = over 80% inhibition of LH release at 72 h but not at 96 h; short = over 80% inhibition of LH release at 3 h but not at 72 h.

(NMP) and then acylated with different amino acids using N^{α}-Boc-Asn/D-Asn/Gln in the presence of *N*,*N*′-diisopropylcarbodiimide (DIC) and 1-hydoxybenzotriazole (HOBt) in NMP. The desired protected resin-bound peptides **2**–**4** were obtained by two steps (i) deprotection of N^{α}-Boc of Asn/D-Asn/Gln with 70% trifluoroacetic acid (TFA) in dichloromethane (DCM); (ii) carbamoylation[10] with *tert*-butyl isocyanate in dimethylformamide (DMF). The analogs **5**–**7** were obtained by the acylation of free ω-amino group of Aph at position 5 with N^{α}-Boc-Dap/D-Dap/Dab(Fmoc) on the otherwise fully protected and assembled peptide resins. The protecting groups on Dap/D-Dap/Dab were removed by sequential treatment with 30% piperidine/NMP and then with 70% TFA/DCM. The carbamoylation of the free amino groups of Dap/D-Dap/Dab with *tert*-butyl isocyanate in dimethylformamide (DMF) gave the desired protected resin-bound peptides **5**–**7**. In analogs **8**–**10**, the coupling of pGlu (in **8**), L-tetrahydro-pyrimidine-2-one-6-carboxylic acid (in **9**) and thymine-1-acetic acid (in **10**) to the free orthogonal amino group at position 5 of Aph was mediated by DIC and HOBt on the resin.

The protected peptido-resins were cleaved and deprotected in anhydrous HF (1.5 h at 0–5 °C) in the presence of anisole. The crude peptides were purified by reversed-phase HPLC (RP-HPLC) in two steps and isolated as their TFA salts.[11] The analytical techniques used for the characterization of the analogs included RP-HPLC with two different solvent systems (acidic and neutral) and capillary zone electrophoresis (CZE). With very few exceptions, all of the analogs were greater than 98% pure. Mass spectrometric analysis supported the identity of the intended structures (Table 1).

All of the GnRH analogs were tested in vitro in a reporter gene assay in HEK-293 cells expressing the human GnRH receptor and a stably integrated luciferase reporter gene as previously described.[12,13] The antagonism of the GnRH induced response by each analog was determined and reported as an IC_{50}, the concentration required to suppress the response in the reporter gene assay by 50% (Fig. 1). In this assay, azaline B (**1**, IC_{50} = 1.36 nM), **8** (IC_{50} = 1.85 nM), and **9** (IC_{50} = 1.78 nM) are the most potent antagonists of the human GnRH receptor with an antagonist IC_{50} below 2.0 nM. Analogs **2** (IC_{50} = 2.22 nM), **3** (IC_{50} = 2.37 nM), **4** (IC_{50} = 6.86 nM), **5** (IC_{50} = 6.37 nM), **6** (IC_{50} = 2.82 nM), **7** (IC_{50} = 3.53 nM) and **10** (IC_{50} = 2.46 nM) were less potent than azaline B. These results clearly indicated that some substitutions at N^{ω}-amino function at position 5 of Aph are well tolerated by the GnRH receptor, however the degree of potency differs depending on the nature, stereochemistry and length of the side chain substitutions (Table 1).

The GnRH antagonists (**1**–**10**) were also tested in vivo in the castrated male rat assay to determine their duration of action at inhibiting LH secretion.[14] In short, test compounds were injected subcutaneously (sc) to castrated male rats at a standard dose of 50 μg/rat. Blood sampling was performed pre-dose and then 3, 72, 96, 120, and 144 h post-dose. The effects of the test compounds on the gonadotropic axis were determined by measurement of plasma LH levels. Most of the analogs with antagonist potency equal to azaline B in vitro were found to be short acting in vivo. Analog **8** (IC_{50} = 1.85 nM) with an L-pyroglutamyl group at position 5 of Aph had a long duration of action similar to that of azaline B and inhibited LH secretion (>80%) for more than 72 h (see Fig. 2).

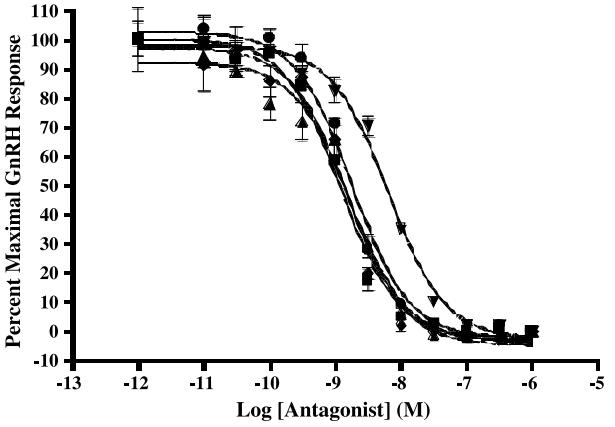

Figure 1. Antagonism of GnRH by selected antagonists in a reporter gene assay in HEK-293 cells expressing the human GnRH receptor and a GnRH-responsive stably integrated luciferase reporter gene. Azaline B: **1**, ■; **2**, ▲; **5**, ▼; **8**, ♦; **9** ●.

The present study was initiated in search of potent GnRH antagonists with longer duration of action and to investigate the possibility of replacing the aminotriaz-

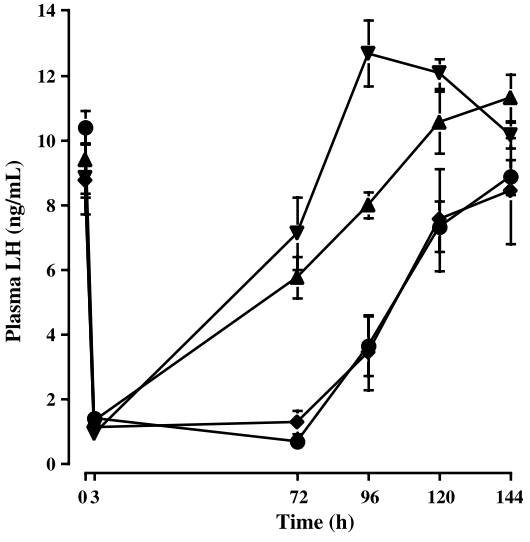

Figure 2. Inhibition of LH secretion in castrated male rats after sc administration of azaline B: **1**, ● and **2**, ▲; **6**, ▼ and **8**, ♦. Total dose was 50 μg/rat in 50 μL of 5% mannitol containing 0.6% DMSO. Blood samples were collected at the times shown on the abscissa (hours). Results are mean plasma LH levels in nanograms per milliliter (n = 6 rats) ± SEM.

olyl moiety in azaline B by synthetically more accessible moieties. N^{α}-Cbm-D- and L-Asn were selected as possible substitutions because of their flexibility and ability to form multiple hydrogen bonds. Remarkably, chirality of the side chains of N^{α}-Cbm-Asn in **2** and N^{α}-Cbm-D-Asn in **3** had no effect on antagonist potency or duration of action. Elongation of the side chain as in N^{α}-Cbm-Gln in **4** resulted in some loss of antagonist potency (IC_{50} = 6.86 nM). Introduction of a carbamoylated dibasic residue such as Dap in **5**, D-Dap in **6** or Dab in **7** had a slightly deleterious effect on antagonist potency as compared to azaline B as well as a significant loss of duration of action. Clearly, the receptor pocket that recognizes these different functionalities and chirality is quite accommodating. This is somewhat distinct from an earlier observation whereby a D-hydroorotyl substitution of Aph^5 was less potent than the L-hydroorotyl derivative (Hor) that ultimately led to the discovery of FE200486 (degarelix) [Ac-D-$2Nal^1$, D-$4Cpa^2$, D-$3Pal^3$, $4Aph(Hor)^5$, D-$4Aph(Cbm)^6$, $ILys^8$, D-Ala^{10}]GnRH.[10] Other acylating agents resembling hydroorotic acid such as pyroglutamic acid in **8**, L-tetrahydro-pyrimidine-2-one)-6-carboxylic acid in **9** and thymine-1-acetic acid in **10** led to significant loss of duration of action as compared to degarelix yet similar in that respect to azaline B in the case of **8**. Analogs **9** and **10** were significantly shorter acting than azaline B.

In summary, most of the new analogs (**2**, **3**, **6**–**10**) are potent antagonists of the human GnRH receptor in vitro with IC_{50} lower than 3.5 nM. This suggests some lack of discrimination by the receptor at position 5. However, with the exception of **8**, they were all short acting in vivo. If we compare the duration of action of any of these analogs with that of degarelix we find the latter to be considerably longer acting. This study therefore allows us to conclude that other parameters than those intrinsically associated to receptor recognition and binding may play a critical role in providing the physicochemical properties responsible for optimal bioavailability and extended duration of action. These properties may include solubility, ability to form reversible gels, and to bind to plasma proteins, resistance to enzymatic degradation or ability to diffuse from the injection site.

Acknowledgements

We thank Ron Kaiser, Charleen Miller for technical assistance and William Low and Dr. Wolfgang Fisher for MS analysis. Work at the Salk Institute was supported by NIH grant HD-39899. In vitro biological assays were carried out at FRI Inc. We thank Debbie Doan for manuscript preparation. J.R. is the Dr. Frederik Paulsen Chair in Neurosciences Professor.

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