Short-chain analogs of luteinizing hormone-releasing hormone containing cytotoxic moieties

(targeted chemotherapeutic agents/receptors on tumors/cancer/peptides)

T. JANÁKY*†, A. JUHÁSZ*, Z. RÉKÁSI*, P. SERFÖZÖ*, J. PINSKI*, L. BOKSER*, G. SRKALOVIC*, S. MILOVANOVIC*, T. W. REDDING‡, G. HALMOS*, A. NAGY*, AND A. V. SCHALLY*‡

Endocrine, Polypeptide and Cancer Institute, †Veterans Affairs Medical Center and *Department of Medicine, Tulane University School of Medicine, New Orleans, LA 70146

Contributed by A. V. Schally, June 26, 1992

ABSTRACT Five hexapeptide and heptapeptide analogs of luteinizing hormone-releasing hormone (LH-RH) were synthesized for use as carriers for cytotoxic compounds. These short analogs were expected to enhance target selectivity of the antineoplastic agents linked to them. Native LH-RH-(3–9) and LH-RH-(4–9) containing D-lysine and D-ornithine at position 6 were amidated with ethylamine and acylated on the N terminus. The receptor-binding affinity of one hexapeptide carrier AJ-41 (Ac-Ser-Tyr-D-Lys-Leu-Arg-Pro-NH-Et) to human breast cancer cell membranes was similar to that of [D-Trp⁶]LH-RH. Alkylating nitrogen mustards (melphalan, Ac-melphalan), anthraquinone derivatives including anticancer antibiotic doxorubicin, antimetabolite (methotrexate), and cisplatin-like platinum complex were linked to these peptides through their ω -amino group at position 6. The hybrid molecules showed no LH-RH agonistic activity *in vitro* and *in vivo* but had nontypical antagonistic effects on pituitary cells *in vitro* at the doses tested. These analogs showed a wide range of receptor-binding affinities to rat pituitaries and cell membranes of human breast cancer and rat Dunning prostate cancer. Several of these conjugates exerted some cytotoxic effects on MCF-7 breast cancer cell line.

More than 2500 agonistic and antagonistic analogs of luteinizing hormone-releasing hormone (LH-RH) have been synthesized since the discovery and structural elucidation (1) of LH-RH in view of their expected medical application (2–4). In addition to the various endocrine and gynecological applications of some of these analogs, they may be potential therapeutic agents in the treatment of hormone-sensitive tumors, like prostate cancer and breast cancer.

The synthetic effort was directed toward finding potent agonists and antagonists by changing amino acids at certain positions, using D-form and/or unusual amino acids. However, only a few reports deal with analogs with shortened LH-RH peptide chain. Deletion of N- or C-terminal amino acids causes a dramatic loss of activity. In 1973–1974 Rivier *et al.* (5, 6) synthesized a series of short-chain LH-RH analogs to find the smallest fragments biologically active by shortening the decapeptide sequence first at the N terminal and, in another series, shortening it at the C terminal. They found that these peptides had only marginal activity, except for [des-Gly¹⁰]LH-RH, which had 10% activity of the parent hormone. Although the free acid analogue of LH-RH exhibited very low potency, replacement of the C-terminal Gly-NH₂ with alkylamides resulted in peptides significantly more active than LH-RH itself (7, 8). Sandow and König (9) examined fragments of LH-RH agonist buserelin ([D-Ser(OBu)⁶, Pro⁹-NH₂]LH-RH where OBu⁹ is *t*-butoxy), and

they found buserelin-(3–9) to be the smallest fragment possessing significant ovulatory activity. Recently, Haviv *et al.* (10) synthesized and tested several reduced-size hexapeptide analogs. Their results showed a wide range of binding affinities (equal to or even exceeding that of LH-RH) to pituitary cell membrane. Depending on the nature of the substituent in positions 4 and 6, the biological responses could be agonistic or antagonistic. Some of their peptides exerted significant antagonistic effect. They suggested that these LH-RH-(4–9) analogs may become useful agents in the treatment of various endocrine disorders.

LH-RH agonists and antagonists with high binding affinity to human breast cancer cell membrane can serve as carriers for cytotoxic compounds (11–13) and target the chemotherapeutic agents to the receptors of cancerous tissues. In the present paper we report the synthesis of reduced-size LH-RH analogs carrying various cytotoxic alkylating agents, anthraquinone derivatives, antimetabolite methotrexate (MTX), and Cisplatin (*cis*-diamminedichloroplatinum)-like platinum complex (Fig. 1).

MATERIALS AND METHODS

Precursor Peptides. Reduced-size LH-RH analogs were synthesized on chloromethyl resin (1 milliequivalent of Cl per g, Bachem) by standard solid-phase peptide synthesis (14) starting with anchoring *tert*-butoxycarbonyl (Boc)-Pro by the KF method (15). Peptides were built step-by-step, coupling Boc-Arg(tosylguanidino), Boc-Leu-, Boc-D-Lys(2-Cl-Z)-, Boc-D-ornithine(Z)-, Boc-Tyr(Cl₂Bzl)-, and Boc-Ser(Bzl)-protected amino acids, where Bzl is benzyl and Z is benzyl-oxycarbonyl (Bachem), using *in situ* preformed 1-hydroxybenzotriazole esters. The N terminus of serine was acylated with Ac-Trp, indole-3-acetic acid (IA), or with 10-fold excess of acetic anhydride. Peptides were cleaved from the resins by treatment with anhydrous ethylamine in dimethylformamide, 1:1 (vol/vol) for 2 days at room temperature. Protective groups were removed by HF (10 ml/g of peptide-resin) at 0°C for 30 min and then at room temperature for an additional 30 min. After elimination of HF, the residue was washed with diethyl ether, and the peptide was dissolved in 50% (vol/vol) aqueous AcOH. The crude product thus obtained was purified by preparative HPLC [Dynamax Macro column (21.2 × 250 mm) packed with spherical C₁₈ silica gel (300-Å pore size, 12-μm particle size) (Rainin, Woburn, MA) using a linear gradient of solvent system *i* consisting of 0.1% aqueous

Abbreviations: LH, luteinizing hormone; LH-RH, LH-releasing hormone; Mel, 4-[bis(2-chloroethyl)amino]phenylalanine; A_{2p}, 2,3-diaminopropionic acid; IA, indole-3-acetic acid (or -acetyl); Boc, *tert*-butoxycarbonyl; MTX, methotrexate; HMAQ, 2-(hydroxymethyl)anthraquinone; DOX, doxorubicin.

†Present address: Department of Medical Chemistry, Albert Szent-Györgyi University Medical School, 6720 Szeged, Dóm tér 8, Hungary.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

FIG. 1. Structures of cytotoxic compounds incorporated into LH-RH analogs. (A) Melphalan. (B) PtCl_2 complex of $\text{A}_{2\text{pr}}$. (C) 2-(Hydroxymethyl)anthraquinone (HMAQ). (D) Doxorubicin (DOX). (E) MTX.

trifluoroacetic acid (A) and 0.1% trifluoroacetic acid in 70% aqueous acetonitrile (B).

Acylation of Ac-Ser-Tyr-D-Lys-Leu-Arg-Pro-NH-Et with $\text{Boc}_2\text{A}_{2\text{pr}}$ (in which $\text{A}_{2\text{pr}}$ is 2,3-diaminopropionic acid), followed by deprotection and purification, resulted in a N^ω -diaminopropionyl-D-lysine derivative. Glutaryl-D-lysine analog was prepared by acylation of Ac-Ser-Tyr-D-Lys-Leu-Arg-Pro-NH-Et with glutaric acid anhydride in the presence of triethylamine in dimethylformamide.

Composition and purity of final precursor peptides were checked by amino acid analysis on a Beckman 6300 amino acid analyzer and reversed-phase analytical HPLC done on Hewlett-Packard model 1090, using a W-Porex 5- μm C_{18} column (4.6 $\times$ 250 mm) and solvent system *i*.

Cytotoxic Compounds. HMAQ, DOX, MTX, cyclopropanecarbonyl chloride, and K_2PtCl_4 were purchased from Aldrich. HMAQ hemiglutarate was prepared by treating HMAQ with glutaric acid anhydride in anhydrous pyridine for 24 hr, and was purified by recrystallization from ethyl acetate/hexane. Melphalan was treated with acetic anhydride to obtain Ac-Mel.

Peptides with Cytotoxic Moiety. Analogs containing 4-[bis(2-chloroethyl)amino]D-phenylalanine (D-Mel) were prepared by treating the free amino group(s) in the side chain of precursor peptides with Boc-D-Mel-O-pentachlorophenyl followed by removal of the Boc group. Ac-Mel was coupled to precursor peptide with 1,3-diisopropylcarbodiimide. Preparation of MTX-containing analog was done by acylation of the free ϵ -amino group of D-lysine in Ac-Ser-Tyr-D-Lys-Leu-Arg-Pro-NH-Et with MTX. The glutamic acid residue of MTX was activated with diisopropylcarbodiimide in dimethylformamide and reacted at 0°C for 10 hr. Anthraquinone derivative was incorporated by preparing the hydroxybenzotriazolyl ester of HMAQ hemiglutarate *in situ* and treating it with the corresponding precursor peptide at 0°C for 24 hr. Ac-Ser-Tyr-D-Lys(glutaryl-DOX)-Leu-Arg-Pro-NH-Et was synthesized by coupling the aminosugar moiety of DOX to the glutaryl side chain of the parent peptide. Hydroxybenzotriazolyl ester of the glutaryl side chain was prepared *in situ* in dimethylformamide by addition of 1,3-diisopropylcarbodiimide, and it was treated with DOX at 0°C overnight. To

obtain metallopeptide containing PtCl_2 , the intermediate peptide having the $\text{A}_{2\text{pr}}$ side chain was treated with K_2PtCl_4 in aqueous dimethylformamide in the presence of sodium acetate for 48 hr at room temperature.

Peptides containing cytotoxic groups were purified on a W-Porex C_{18} column (10 $\times$ 250 mm; pore size, 300 Å; particle size, 5 μm) (Phenomenex, Rancho Palos Verdes, CA) using solvent system *i* or solvent system *ii* consisting of 0.2% aqueous acetic acid (A) and 0.2% acetic acid in 70% aqueous acetonitrile (B) in a gradient mode. Column eluant was monitored with UV detectors operating at 230 or 280 nm. Chromatography was done at ambient temperature.

LH-releasing and LH-RH-inhibiting activities. The ability of compounds to influence LH release *in vitro* was assayed by using a dispersed rat pituitary cell superfusion system (16).

LH-releasing effect was determined by perfusing the analogs through the cells for 3 min in a 1-ml vol of perfusate at 0.1- to 10-nM concentrations. LH content of 1-ml fractions collected was determined by RIA. The potency of peptides was compared to that of 3 nM LH-RH perfused in the same way.

LH-RH inhibiting effect of analogs was tested as follows: each peptide was perfused through the cells for 9 min in a 3-ml vol at 10 or 30 nM concentrations. Next, a mixture containing the same concentration of analog and 3 nM LH-RH was immediately administered for 3 min. This step was followed by four consecutive infusions of 3 nM LH-RH for 3 min (1 ml of perfusate) at 30-min intervals (30, 60, and 90 min). LH content of the 1-ml fractions collected was determined by RIA.

In vivo test. Male rats weighing 380–450 g were castrated 3 weeks before the experiment, anesthetized with Metofane, and injected s.c. with antagonist [Ac-D-Nal(2)¹, D-Phe(4Cl)², D-Pal(3)³, D-Cit⁶, D-Ala¹⁰]LH-RH (SB-75) [where Nal(2) is 3-(2-naphthyl)alanine, Pal(3) is 3-(3-pyridyl)alanine, and Cit is citrulline (2-amino-5-ureidovaleric acid)] at a dose of 5 μg per rat and with analog AJ-41 (for structure, see below) in doses of 50 and 500 μg per rat. Blood samples were taken from the jugular vein before and 2, 4, 24, and 48 hr after administration of the peptides for LH measurement.

Receptor Binding. The affinity for the LH-RH receptors of rat pituitary, human breast cancer, and rat Dunning 3227 cell membranes was determined by using [¹²⁵I]-labeled [D-Trp⁶]LH-RH as radioactive ligand (17).

Cytotoxicity Test. The ability of peptides with cytotoxic moieties to inhibit incorporation of [³H]thymidine into DNA in monolayer cultures of the human mammary tumor cell line MCF-7 was assayed as described (18).

RESULTS

Preparation of Reduced-Size LH-RH Analogs. Peptide analogs I, III, VI, and XII containing LH-RH-(4-9) were synthesized by solid-phase technique. The N terminal was blocked by acetylation, reaction with IA, or by elongation of the peptide chain with Ac-Trp. Replacement of the C-terminal Gly-NH₂ of LH-RH with an ethylamide group resulted in peptides with the general formula of R¹-Ser-Tyr-R⁴-Leu-Arg-Pro-NH-Et (in which, R¹ is acetyl, Ac-Trp, or IA; and R⁴ is D-lysine or D-ornithine).

To facilitate the incorporation of some chemotherapeutic agents into position R⁴, peptide XII was derivatized at that position either by acylation of its D-lysine residue with glutaric anhydride (peptide XIV) or with 2,3-diaminopropionic acid (peptide XIII). After purification by preparative HPLC, the purity of the isolated peptides was >96%. Hydrophobicity of the series was assessed by the capacity factor (*K'*) obtained on analytical HPLC (Table 1). *K'* values of carriers containing indole ring at the N terminal (peptides I, III, VI, and IX) were seven times higher than that of peptide XII. IA or Ac-Trp substitution changed the hydrophobicity

Table 1. HPLC capacity factor and receptor-binding affinity of R¹-Ser-Tyr-R⁴(Q)-Leu-Arg-Pro-NH-Et peptides containing cytotoxic radicals to cell membranes of human breast cancers

No.	Code	Peptide				Affinity constant,† nM ⁻¹
		R ¹	R ⁴	Q	<i>K'</i> *	
I	AJ-40	IA	D-Lys		4.31	0.11
II	AJ-48	IA	D-Lys	D-Mel	13.36	
III	AJ-43	IA	D-Orn		4.10	0.23
IV	AJ-52	IA	D-Orn	D-Mel	13.36	
V	T-153	IA	D-Orn	Glt-HMAQ	17.45	0.025
VI	AJ-42	Ac-Trp	D-Lys		4.59	NB
VII	AJ-47	Ac-Trp	D-Lys	D-Mel	13.32	NB
VIII	T-152	Ac-Trp	D-Lys	Glt-HMAQ	17.41	
IX	AJ-44	Ac-Trp	D-Orn		4.39	NB
X	AJ-53	Ac-Trp	D-Orn	D-Mel	14.71	
XI	T-154	Ac-Trp	D-Orn	Glt-HMAQ	17.28	
XII	AJ-41	Ac	D-Lys		0.59	1.6
XIII	AJ-55	Ac	D-Lys	A ₂ pr	1.33	
XIV	T-173	Ac	D-Lys	Glt	3.04	NB
XV	AJ-50	Ac	D-Lys	D-Mel	10.87	
XVI	AJ-51	Ac	D-Lys	MTX	4.67	0.83
					4.84	
XVII	AJ-62	Ac	D-Lys	Ac-Mel	11.04	0.003
XVIII	T-172	Ac	D-Lys	Glt-HMAQ	15.28	0.04
XIX	T-177	Ac	D-Lys	Glt-DOX	11.53	0.48
XX	T-175	Ac	D-Lys	A ₂ pr(PtCl ₂)	4.55	0.87
XXI	T-174	Ac	D-Lys	A ₂ pr-(Glt-HMAQ) ₂	13.16	0.48

HPLC system was as follows: 4.6 × 250 mm C₁₈ column; eluent A, 0.1% trifluoroacetic acid; eluent B, 70% MeCN/0.1% trifluoroacetic acid; gradient was 20–60% B in 40 min at 1.2 ml/min. Orn, ornithine; NB, no binding; Glt, glutaryl.

*Capacity factor, *K'* = (retention volume – void volume)/void volume.

†Affinity constants were determined by using [¹²⁵I]-labeled [D-Trp⁶]LH-RH as radioactive ligand.

Table 2. Receptor-binding affinity of peptides without cytotoxic radicals to rat hypophysis and rat Dunning prostate cell membranes, as determined by using [¹²⁵I]-labeled [D-Trp⁶]LH-RH

Peptide	Affinity constant*			
	Hypophysis		Dunning tumor	
	K _{a1} , nM ⁻¹	K _{a2} , μM ⁻¹	K _{a1} , nM ⁻¹	K _{a2} , μM ⁻¹
A†	0.44	2.34	1.40	2.20
I	0.69	1.13	5.70	0.54
III	0.45	0.69	22.05	114.16
VI	6.11	16.40	23.94	15.79
IX	0.45	0.52	5.02	4.07
XII	7.02	3.99	NB	NB

NB, no binding.

*K_{a1} and K_{a2} are affinity constants of peptides for the first and second receptor class (i.e., high- and low-affinity binding sites).

†[D-Trp⁶]LH-RH.

equally, whereas replacing D-lysine with D-ornithine did not cause significant changes.

Receptor-Binding Affinities of These Analogs. These affinities were evaluated not only with rat pituitary membranes but also with human breast cancer and rat Dunning prostate cancer cell membranes (Tables 1 and 2) because the analogs were intended to carry (transport) cytotoxic compounds to receptors on cancerous tissues. Binding affinities of the carrier molecules to pituitary receptors were similar to that of the control [D-Trp⁶]LH-RH. Affinity of peptide carrier XII to human breast cancer cell membrane was relatively high. Although its cytotoxic analogs (peptide XVI > peptide XIX > peptide XVII) showed somewhat smaller affinity, they still preserved significant binding properties. Incorporation of glutaric acid (peptide XIV) abolished the binding to breast cancer cell membrane receptors.

Endocrine Effects of These Analogs. Table 3 shows that the analogs did not function as true antagonistic compounds. The increase in the inhibitory effect on the LH response with time with peptides VI, XII, and XIII is probably from secondary processes. The analogs showed no agonistic activity *in vitro*

Table 3. LH-RH antagonistic activity of R¹-Ser-Tyr-R⁴(Q)-Leu-Arg-Pro-NH-Et peptides (see Table 1) in perfused rat pituitary cell system and their inhibitory effect on incorporation of [³H]thymidine in MCF-7 human mammary cancer cell line

Peptide	Dose, nM	LH response				Cytotoxic inhibition, %	
		Inhibition, %				0.5 μM dose	5 μM dose
		0 min	30 min	60 min	90 min		
II	10	15	32	41	44		
III	10	0	11	12	0	41	45
IV	10	12	33	30	41	36	50
V	30	14	21	32	—	33	47
VI	10	0	18	31	38		
VII	10	3	4	14	21		
VIII	30	18	3	14	—		
IX	10	3	7	15	0	36	45
X	10	13	33	33	36		
XI	30	19	22	33	—	26	55
XII	10	0	12	20	27	33	57
XIII	10	6	20	21	31	24	29
XV	10	1	11	22	32		
XVI	30	0	0	0	0	33	22
XVII	—	—	—	—	—	38	48
XVIII	30	0	0	0	—	33	57
XIX	30	0	3	29	—	40	53
XX	30	14	19	37	—		
XXI	30	2	8	13	—	19	40

at the doses tested (0.1–10 nM). One of the short-chain LH-RH analogs, AJ-41, was selected for *in vivo* tests in castrated rats and compared with a well-known LH-RH antagonist SB-75. Analog AJ-41 showed no LH-suppressing activity at a dose of 500 μ g per rat, whereas analog SB-75 produced a 100% LH inhibition at a dose of 5 μ g per rat (Fig. 2). As expected, analog AJ-41 did not induce LH release at this dose, which is 1000 times higher than the active dose of LH-RH.

Fig. 1 shows the cytotoxic compounds that were coupled to carrier peptides. ϵ -Amino side-chain group at position 4 was acylated directly with melphalan, acetyl melphalan, and MTX, whereas HMAQ and DOX were connected through a bridge molecule (glutaric acid). Platinum complex (peptide XX) was formed by chelating platinum with the two amino groups of the A_{2pr} side chain of peptide XIII. The same starting compound was used to incorporate two molecules of HMAQ (peptide XXI). Peptide XII was the most hydrophilic peptide among our carrier compounds, and its derivatives preserved this character (Table 1)—that is, the capacity factors of cytotoxic conjugates with the backbone of peptide XII were lower than those of the other peptide bearing the same substituents (peptide II > peptides XV and peptide V > peptide XVIII). Nonspecific activation of the two carboxyl groups of the glutaric acid moiety in MTX resulted in two isomeric conjugates (peptide XVI) with carrier XII. Although the high resolution of our HPLC system enabled us to detect the two isomers, their separation was not done.

A similar nontypical inhibition of LH response was found when peptides with cytotoxic moieties were tested for antagonistic activity (Table 3). Nearly all the compounds showed increased inhibitory effect with time; however, none of them exerted significant antagonistic activity at 0 min when infused at relatively high 10 to 30 nM concentrations.

The effect of analogs on [3 H]thymidine incorporation was evaluated in cell cultures of MCF-7 human breast cancer cell line (Table 3). All analogs tested reduced [3 H]thymidine incorporation, especially peptide XII; peptides XVIII and XIX also had high cytotoxic activity.

DISCUSSION

Although a large number of LH-RH analogs were made, only a few shortened peptides were synthesized and investigated for their biological potencies (5, 6, 9). With few exceptions (7, 8), these shortened analogs had low LH-RH agonistic activity. To evaluate *in vitro* potencies and to study the structure-activity relationships, 29 hexapeptide and heptapeptide analogs with agonistic or antagonistic activity were synthesized by Haviv *et al.* (10). Evaluation of modifications in positions 1 and 4 of these analogs (positions 3 and 6 of LH-RH) led to the conclusions that the delicate balance between agonistic

and antagonistic activity is influenced by substituents in these positions. Their results suggest that there is an optimal size, length, and shape for substituents at position 1 for achieving affinity, potency, and agonistic versus antagonistic activity (10). Our peptide carriers had no LH-releasing activity or true LH-RH inhibiting activity at the doses tested. Binding affinity of these hexapeptide analogs, produced by replacing IA^1 with $Ac-Trp^1$ (peptides I–IX), depended on the substituent in position 4 and on the type of tissue membrane examined. The relatively high binding affinity of compound XII to breast cancer cell membranes and good binding to pituitary cell membranes is noteworthy, considering that compound XII contains only six amino acids and no bulky substituent in position 1. The Gly^6 replacement in LH-RH antagonists by D-amino acid residues led to peptides with enhanced antagonistic potency (2, 3, 19).

We incorporated D-lysine or D-ornithine into position 4 (position 6 in LH-RH), which resulted in peptides with a free amino group in the side chain. Changing D-lysine to D-ornithine in analogs with IA at position 1 (peptides I and III) increased the receptor-binding affinity to human breast cancer receptors, whereas in the case of analogs with $Ac-Trp$ at position 1 (peptides VI and IX), we found no binding (Tables 1 and 2). We could demonstrate differences between the nontypical LH-RH inhibition by peptides containing D-Lys 4 (peptides VI, XII, and XIII) and D-Orn 4 (peptides III and IX) (Table 3). Although peptides VI, XII, and XIII each increased inhibition, peptides with one less CH_2 in the side chain of position 4 (i.e., D-ornithine) had only marginal activity.

LH-RH agonists can inhibit the growth of estrogen-dependent mammary tumors in women with breast cancer (20, 21) by inducing a state of ovarian hormone ablation. Much evidence suggests that, in addition to the inhibitory effect on the pituitary–gonadal axis, some of these analogs may act directly on tumor cells (22, 23). Hormones are known to exert their effect through binding to specific receptors on their target cells. Thus hormone–drug conjugates could provide a viable approach for site-specific delivery of cytotoxic agents to tumors, although their mechanism of action is not well understood. Combination of hormonal manipulation with targeted chemotherapy could enhance the efficacy of the treatment. LH-RH analogs might serve as carriers for chemotherapeutic agents by proposed mechanisms (13).

From our recent studies on LH-RH analogs with cytotoxic moieties (11–13, 24), we selected those cytotoxic compounds that proved useful for coupling to the side chain of LH-RH agonists and antagonists (Fig. 1). Cytotoxic effects of shortened-chain LH-RH analogs and their conjugates with anticancer drugs were investigated in human MCF-7 breast cancer cell lines. Despite the fact that our carriers do not have any agonistic or characteristic antagonistic activity, they inhibited [3 H]thymidine incorporation. The basis for this antitumor activity *in vitro* is not understood because these carrier analogs have no hormonal activity. LH-RH-like peptides have been postulated to be produced by some tumors and may act locally as growth factors (17, 25, 26). The short-chain carrier LH-RH analogs in high doses might interfere with these LH-RH-like peptides. This possible phenomenon must be investigated. We have previously established that decapeptide LH-RH analogs with cytotoxic substituents show high antitumor activity *in vitro* (13), as well as *in vivo* (24). The cytotoxic activity of the short-chain carrier analogs must be also investigated *in vivo* and compared with their analogs with cytotoxic radicals. It is unlikely that short carriers can inhibit the pituitary–gonadal axis and thus cause sex-steroid deprivation. “Short” cytotoxic analogs would be easier to test because their short carriers should have no biological and probably no oncological activity *in vivo*, in contrast to long-decapeptide carriers.

FIG. 2. Serum LH levels in castrated male rats after s.c. injection of the short-chain LH-RH analog AJ-41 in doses of 50 and 500 μ g per rat and of the LH-RH antagonist SB-75 at a dose of 5 μ g per rat.

Hexapeptide carrier AJ-41 had no endocrine effects *in vivo* on LH release in doses 1000 times greater than LH-RH (Fig. 2). To elucidate these complex effects and to discover the mechanism of action of these short-chain cytotoxic LH-RH analogs and their carriers will require extensive additional studies both *in vivo* and *in vitro*.

The work described in this paper was supported by National Institutes of Health Grants CA-40003 and CA-40004 and by the Medical Research Service of the Veterans Affairs (to A.V.S.).

1. Matsuo, H., Baba, Y., Nair, R. M., Arimura, A. & Schally, A. V. (1971) *Biochem. Biophys. Res. Commun.* **43**, 1334–1339.
2. Karten, M. J. & Rivier, J. E. (1986) *Endocr. Rev.* **7**, 44–66.
3. Dutta, A. S. (1988) *Drugs Future* **13**, 761–787.
4. Dutta, A. S. (1988) *Drugs Future* **13**, 43–57.
5. Rivier, J. E., Vale, W., Burgus, R., Amoss, M., Blackwell, R. & Guillemin, J. (1973) *J. Med. Chem.* **16**, 545–549.
6. Rivier, J. E., Amoss, M. & Rivier, C. (1974) *J. Med. Chem.* **17**, 230–233.
7. Sievertsson, H., Chang, J. K., Bogentoft, C., Currie, B. L. & Folkers, C. Y. (1971) *Biochem. Biophys. Res. Commun.* **44**, 1566–1571.
8. Fujino, M., Kobayashi, S., Obayashi, M., Shinagawa, S., Fukuda, T., Kitada, C., Nakayaga, R., Yamazaki, I., White, W. F. & Rippel, R. H. (1972) *Biochem. Biophys. Res. Commun.* **49**, 863–869.
9. Sadow, J. & König, W. (1979) *J. Endocrinol.* **81**, 175–182.
10. Haviv, F., Palabrica, C. A., Bush, E. N., Diaz, G., Johnson, E. S., Love, S. & Geer, J. (1989) *J. Med. Chem.* **32**, 2340–2344.
11. Bajusz, S., Janáky, T., Csernus, V., Bokser, L., Fekete, M., Srkalovic, G., Redding, T. W. & Schally, A. V. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6318–6322.
12. Bajusz, S., Janáky, T., Csernus, V., Bokser, L., Fekete, M., Srkalovic, G., Redding, T. W. & Schally, A. V. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6313–6317.
13. Janáky, T., Juhasz, A., Bajusz, S., Csernus, V., Srkalovic, G., Bokser, L., Milovanovic, S., Redding, T. W., Rekasi, Z., Nagy, A. & Schally, A. V. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 972–976.
14. Stewart, J. M. & Young, J. D. (1984) *Solid Phase Peptide Synthesis* (Pierce, Rockford, IL), 2nd Ed.
15. König, W. & Geiger, R. (1971) *Ber. Dtsch. Chem. Ges.* **103**, 788–798.
16. Csernus, V. & Schally, A. V. (1991) in *Neuroendocrine Research Methods*, ed. Greenstein, B. (Harwood, London), pp. 71–109.
17. Fekete, M., Bajusz, S., Groot, K., Csernus, V. & Schally, A. V. (1989) *Endocrinology* **124**, 946–955.
18. Sondak, V. K., Bertelsen, C. A., Tanigawa, N., Hildebrand-Zanki, S. U., Morton, D. L., Korn, E. L. & Kern, D. H. (1984) *Cancer Res.* **44**, 1725–1728.
19. Bajusz, S., Janáky, T., Csernus, V. J., Fekete, M., Bokser, L. & Schally, A. V. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 1637–1641.
20. Schally, A. V., Bajusz, S., Redding, T. W., Zalutnai, A. & Comaru-Schally, A. M. (1989) in *GnRH Analogues in Cancer and in Human Reproduction*, eds. Vickery, V. H. & Lunenfeld, B. (Kluwer, Dordrecht, The Netherlands), Vol. 1, pp. 5–31.
21. Nicholson, R. I., Walker, K. J., Walker, R. F., Read, G. F., Turkes, A., Robertson, J. F. R. & Blamey, R. W. (1989) *Horm. Res.* **32**, 198–201.
22. Blankeinstein, M. A., Hankelman, M. S. & Klijn, J. G. (1985) *Eur. J. Cancer Clin. Oncol.* **21**, 1493–1499.
23. Miller, W. R., Scott, W. N., Morris, R., Fraser, H. M. & Sharpe, R. M. (1985) *Nature (London)* **313**, 231–233.
24. Szepeshazi, K., Schally, A. V., Milovanovic, S., Juhasz, A., Nagy, A. & Janáky, T. (1992) *Anticancer Drugs* **3**, 109–116.
25. Quayum, A., Gullick, W., Clayton, R. C., Sikora, K. & Waxman, J. (1990) *Br. J. Cancer* **62**, 96–99.
26. Harris, N., Dutlow, S., Eidne, K., Dong, K.-W. & Millar, R. (1991) *Cancer Res.* **51**, 2577–2581.