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Inhibition of growth of a prolactin and growth hormone-secreting pituitary tumor in rats by D-tryptophan-6 analog of luteinizing hormone-releasing hormone

(pituitary GH_3 tumor/analogs of hypothalamic hormones)

I. TORRES-ALEMAN, T. W. REDDING, AND A. V. SCHALLY

Endocrine and Polypeptide Laboratories, Veterans Administration Medical Center, and Department of Medicine, Tulane University School of Medicine, New Orleans, LA 70146

Contributed by A. V. Schally, October 15, 1984

ABSTRACT **The effect of long-term administration of analogs of luteinizing hormone-releasing hormone (LH-RH) and somatostatin on the growth of the growth hormone (GH)- and prolactin (PRL)-secreting rat pituitary GH_3 tumor was investigated. Daily administration of [D-Trp^6]LH-RH (50 μg/day), early after inoculation of the GH_3 tumor, inhibited tumor growth by more than 90% as compared to controls. Similarly, in two experiments, a single once-a-month injection of long-acting [D-Trp^6]LH-RH microcapsules (in a dose calculated to release about 25 μg/day for 30 days) inhibited the growth of GH_3 pituitary tumor by more than 50% 6 or 13 wk after transplantation, when the tumors were fully developed. Serum GH and PRL levels also were reduced markedly by treatment with [D-Trp^6]LH-RH. On the other hand, the administration of an antagonistic analog of LH-RH, *N*-Ac-[D-Phe(4Cl)^{1,2}, D-Trp^3, D-Arg^6, D-Ala^{10}]LH-RH, did not significantly reduce the growth of this tumor, and the treatment with two different analogs of somatostatin, cyclo(Pro-Phe-D-Trp-Lys-Thr-Phe) and D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr NH_2, appeared to enhance it. These results are in agreement with previous findings of growth inhibition of 7315a pituitary tumors with different hormone-secreting characteristics by agonistic analogs of LH-RH. The collective data from experimental work with rat pituitary tumor models support the contention that the use of [D-Trp^6]LH-RH might be considered for the treatment of some patients with pituitary tumors who failed to respond to conventional therapy.**

Previous extensive experimental and clinical studies have demonstrated a potential use of the D-tryptophan-6 analog of luteinizing hormone-releasing hormone (LH-RH) for the treatment of various endocrine-dependent tumors (1). This highly active agonistic analog of LH-RH has been shown to exert paradoxical inhibitory effects on the pituitary–gonadal axis in both animals and human beings when given chronically (2, 3). This inhibition of reproductive functions produced by chronic administration of [D-Trp^6]LH-RH and other LH-RH agonists has been utilized to induce a regression of various hormone-sensitive neoplasms such as mammary carcinomas, prostate tumors, and pituitary tumors (4–7). It has been shown that long-term administration of agonistic and antagonistic analogs of LH-RH inhibited the growth of the 7315a transplantable rat pituitary tumor, which secretes both prolactin (PRL) and corticotropin (ACTH) (6, 7). In order to study further the inhibitory effect of [D-Trp^6]LH-RH on pituitary tumors, we decided to extend our investigations to the PRL- and growth hormone (GH)-producing GH_3 rat pituitary tumor. This transplantable pituitary tumor is a clonal strain derived from an ACTH/GH-secreting tumor (8). It shares many characteristics of normal pituitary cells but responds poorly to therapy with dopamine agonists such as 2-bromo-α-ergocryptine (called bromocriptine) (9). Treatment of some human pituitary tumors with bromocriptine does not invariably result in clinical improvement, suggesting an insensitivity of this type of tumor to DA agonists therapy (10). Thus, an investigation of the growth inhibition of different rat pituitary tumors by [D-Trp^6]LH-RH could lead to findings of clinical importance. A part of this study was reported previously in abstract form.*

MATERIALS AND METHODS

Peptides. [D-Trp^6]LH-RH was synthesized by solid-phase methods and supplied by Debiopharm (Lausanne, Switzerland). Microcapsules of [D-Trp^6]LH-RH were prepared by a phase-separation process and supplied by T. Tice, Southern Research Institute (Birmingham, AL). The product was a free-flowing powder of spherical particles consisting of [D-Trp^6]LH-RH (2% wt/wt), distributed within a polymeric matrix of 53:47 (mol %) poly(D,L-lactide-co-glycolide) (98% wt/wt). These microcapsules were designed for continuous controlled release of this peptide over a period of 30 days (11). N-Ac-[D-Phe(4Cl)^{1,2}, D-Trp^3, D-Arg^6, D-Ala^{10}]LH-RH (LH-RH antagonist) (ORG 30276) was obtained from Organon (Oss, Holland). Somatostatin analog cyclo(Pro-Phe-D-Trp-Lys-Thr-Phe) (Veber cyclic hexapeptide) (12) was synthesized in our laboratory or supplied by J. Sandow and R. Geiger (Hoechst, Frankfurt M, FRG). Somatostatin analog D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2 related to the octapeptide of Bauer (13) was synthesized in our laboratory by solid-phase methods described previously (14).

Female Wistar/Furth rats (80–100 g, Harlan Sprague–Dawley, Indianapolis, IN) were inoculated s.c. in the scapular region with GH_3 pituitary tumor cells (obtained from the American Type Culture Collection). The animals were housed 5–7 per cage in a temperature- and light-controlled room. Five to seven animals were used per experimental group. In the first experiment, the treatment with peptides was started before tumors appeared, while in subsequent experiments, peptide administration was initiated when 50–100% of the animals showed well-developed tumors. Tumors were measured weekly with microcalipers and tumor volumes were calculated as described (7).

Experiment I. Three days after inoculation of 1×10^6 GH_3

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Abbreviations: LH, luteinizing hormone; LH-RH, luteinizing hormone-releasing hormone; PRL, prolactin; GH, growth hormone; b.i.d., twice a day; ACTH, corticotropin.

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Table 1. Effect of early administration of analogs of LH-RH and somatostatin (SS) on tumor volume and on tumor and organ weight in female Wistar/Furth rats bearing GH_3 pituitary tumor

Treatment*	Dose b.i.d., μg	Final tumor volume		Weight		
		mm³	%	Tumor, g	Ovarian, mg	Adrenal, mg
Control		538 ± 230	100	0.52 ± 0.2	64.6 ± 4	44 ± 2
[D-Trp⁶]LH-RH	25	34.5 ± 15†	6.4	0.04 ± 0.02†	14.4 ± 1‡	62 ± 5‡
Veber cyclic§ hexapeptide SS	5	894.8 ± 399	166	1.04 ± 0.5	58 ± 6	52 ± 4
Modified Bauer¶ SS octapeptide	5	1041 ± 454	193	1.13 ± 0.5	59 ± 3	53 ± 4

*Treatment with the analogs was started 3 days after inoculation of GH_3 tumor cells to the animals and was continued for 7 wk.
†$P < 0.05$ vs. control by Student's t test.
‡$P < 0.005$ vs. control by Student's t test.
§Cyclo(Pro-Phe-D-Trp-Lys-Thr-Phe).
¶D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2.

cells per animal, the treatment with three different peptides was started. Group I received 10% polyvinylpyrrolidone/1% propylene glycol in saline. Group II was injected with [D-Trp⁶]LH-RH (25 μg) twice a day (b.i.d.) in 10% polyvinylpyrrolidone/1% propylene glycol in saline. Group III was treated with Veber cyclic hexapeptide (5 μg b.i.d.), and group IV, with Bauer-modified octapeptide (5 μg b.i.d.), both in the same vehicle. The treatment lasted 7 wk.

Experiment II. Six weeks after inoculation of 5×10^5 GH_3 cells per animal, 50% of the rats developed palpable tumors. Treatment with the peptides was initiated at the beginning of week 6 and continued for 1 month. Group I received the injection vehicle. Group II was treated with [D-Trp⁶]LH-RH microcapsules. Group III received the LH-RH antagonist (50 μg b.i.d.). The microcapsules, in aliquots of 33 mg calculated to release a dose of about 25 μg/day for 30 days, were suspended in disposable syringes in 0.7 ml of injection vehicle containing 2% (wt/vol) carboxymethylcellulose and 1% Tween 20 in water. The suspension was mixed thoroughly on a Vortex mixer and injected through an 18-gauge needle deep into the thigh muscle of rats.

Experiment III. Thirteen weeks after inoculation of 1×10^5 GH_3 cells per animal, when all the animals had well-developed tumors, treatment with [D-Trp⁶]LH-RH microcapsules in the same dose as in experiment II was started. Controls received the injection vehicle. The rats were sacrificed after 30 days.

All of the peptides were injected subcutaneously at 9:00 a.m. and 4:30 p.m., daily. The microcapsules of [D-Trp⁶]LH-RH and its injection vehicle were given i.m. once a month. The animals were sacrificed 2–4 hr after the last injection of peptides (30 days in the case of microcapsules), and trunk blood was collected. Tumor, body, pituitary, ovaries, and adrenal weights were recorded, and tumor volume was measured (7). Pituitary tissue was homogenized in distilled water and centrifuged (4000 rpm for 30 min at 4°C; Sorvall H 4000 rotor), and the supernatants were stored. Serum and pituitary extracts were kept at −20°C until assayed.

All data were expressed as means ± SEM. Statistical analyses were made by Student's t test or nonparametric rank-sign test of Wilcoxon (15).

RIA. Pituitary hormone and serum levels were measured by RIA. GH, PRL, and LH RIAs were done with materials supplied by the National Hormone and Pituitary Program. GH RP-1, PRL RP-III, and LH RP-II were used as reference preparations. Interassay coefficient of variation was 15% or less, and intraassay coefficient of variation was 10% or less for all three hormones. Insulin RIA was performed with a commercial kit (Cambridge Medical Diagnostics, Billerica, MA), and progesterone and 17-β-estradiol RIAs were done with commercially available reagents (Radioassay Systems Laboratories, Carson, CA).

RESULTS

The effects of administration of [D-Trp⁶]LH-RH and analogs of somatostatin on the growth of pituitary GH_3 tumor were first evaluated by starting the treatment 3 days after inoculation. Table 1 shows the effect of [D-Trp⁶]LH-RH and two somatostatin analogs on tumor volume and tumor and organ weights in female rats bearing GH_3 pituitary tumor. Animals treated with [D-Trp⁶]LH-RH had very small or no palpable tumors. Treatment with [D-Trp⁶]LH-RH resulted in a more than 90% reduction in tumor volume and weight as compared to controls ($P < 0.05$). [D-Trp⁶]LH-RH treatment diminished ovarian weights and increased adrenal weight (Table 1), while pituitary weights were not affected (not shown). On the other hand, treatment with both of the somatostatin analogs enhanced tumor growth, with tumor volume and weight being increased by at least 40% over control values (Table 1). These findings are in agreement with hormone levels found in these animals. Table 2 shows serum and pituitary levels of PRL, GH, and LH. Serum PRL and GH levels and pituitary PRL and LH concentrations were greatly reduced by the administration of [D-Trp⁶]LH-RH ($P < 0.05$ and $P < 0.005$, respectively), while serum LH levels were increased after this same treatment ($P < 0.01$). Conversely, administration of either Veber somatostatin cyclohexapep-

Table 2. Effect of administration of [D-Trp⁶]LH-RH and analogs of somatostatin (SS) on pituitary and serum levels of PRL, GH, and LH in female Wistar/Furth rats bearing pituitary GH_3 tumor

Treatment	PRL in		GH in		LH in	
	Serum, ng/ml	Pituitary, ng/mg	Serum, ng/ml	Pituitary, μg/mg	Serum, pg/ml	Pituitary, ng/mg
Control	59.4 ± 24	217 ± 28	395 ± 158	138 ± 27	696 ± 79	511 ± 57
[D-Trp⁶]LH-RH	3.2 ± 0.7*	73 ± 7†	64 ± 16*	150 ± 34	2684 ± 140†	42 ± 6†
Veber cyclic SS hexapeptide	212 ± 112	214 ± 40	862 ± 308	61 ± 25	571 ± 83	380 ± 77
Modified Bauer SS octapeptide	181 ± 76	224 ± 65	972 ± 406	34 ± 9	635 ± 101	458 ± 190

*$P < 0.05$ vs. control by Student's t test.
†$P < 0.005$ vs. control by Student's t test.

Table 3. Effect of early administration of [D-Trp^6]LH-RH on serum levels of progesterone, 17-β-estradiol, and insulin in female Wistar/Furth rats bearing pituitary tumor GH_3

Treatment	Progesterone, ng/ml	17-β-estradiol, pg/ml	Insulin, microunits/ml
Control	73.3 ± 26.8	107.5 ± 10.6	27 ± 2
[D-Trp^6]LH-RH	8.7 ± 2.1*	57.2 ± 5.8†	19 ± 0.6‡

*$P < 0.05$ vs. control by Student's t test.
†$P < 0.01$ vs. control by Student's t test.
‡$P < 0.005$ vs. control by Student's t test.

tide (Pro-Phe-D-Trp-Lys-Thr-Phe) or modified Bauer somatostatin octapeptide D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-NH_2 produced an increase in serum PRL and GH levels and decreased pituitary GH levels. Treatment with [D-Trp^6]LH-RH also reduced serum progesterone, 17-β-estradiol, and insulin levels in these animals ($P < 0.05$, $P < 0.01$, and $P < 0.005$, respectively) (Table 3).

The effect of administration of LH-RH analogs on rats bearing well-developed GH_3 tumors was investigated next. The treatment was started when 50% of the animals had well-developed tumors. Administration of [D-Trp^6]LH-RH microcapsules resulted in a reduction of tumor growth of more than 50%, as indicated by the reduced increment in tumor volume ($P < 0.05$), decreased tumor weight ($P = 0.05$) (Table 4, experiment II), and lower tumor incidence (67%). Ovarian weights were again decreased by [D-Trp^6]LH-RH administration, while pituitary, body, and adrenal weights were not affected (not shown). Administration of the LH-RH antagonist N-Ac-[D-Phe(4Cl)^{1,2}, D-Trp^3, D-Arg^6, D-Ala^{10}]LH-RH at a dose that suppressed the ovarian weights to the same extent as [D-Trp^6]LH-RH (see Table 4, experiment II) produced only a small and not significant decrease in tumor volume and weight and caused no change in tumor incidence rate. In agreement with the results of experiment I (Table 2), serum PRL and GH levels and pituitary PRL and LH concentrations were greatly reduced by treatment with [D-Trp^6]LH-RH as compared to controls (Table 5). Pituitary GH levels were increased by [D-Trp^6]LH-RH administration, while serum LH levels were similar to control values. On the other hand, treatment with the LH-RH antagonist produced a small but significant decrease in serum LH and minor decreases in serum GH and PRL levels and pituitary LH concentrations (Table 5).

In another experiment, the [D-Trp^6]LH-RH microcapsules were again administered when all the animals had well-developed tumors. Tumor growth was once more inhibited by treatment with [D-Trp^6]LH-RH microcapsules as demonstrated by a more than 60% reduction in final tumor volume and tumor weight as compared with controls (Table 4, experiment III). The increment in tumor volume for the group treated with microcapsules was decreased by 67%. Ovarian weights were reduced by injection of [D-Trp^6]LH-RH microcapsules. The hormone levels in this experiment were similar to those obtained in experiment II of Table 5. These results confirmed that once-a-month injection of [D-Trp^6]LH-RH microcapsules can inhibit pituitary GH_3 tumor growth by at least 50%.

DISCUSSION

The results of this study with GH_3 pituitary tumors confirm and extend our previous work on 7315a tumors and suggest a potential use of [D-Trp^6]LH-RH for the treatment of pituitary tumors (1, 2). [D-Trp^6]LH-RH administration caused an almost complete inhibition (over 90%) of the growth of pituitary tumor GH_3 when treatment was initiated early in the development of the tumors. Tumor growth was also markedly reduced (over 50%) when injections of the analog were started after the tumors were well-developed. Serum PRL and GH levels were decreased by treatment with [D-Trp^6]LH-RH. Thus, chronic administration of [D-Trp^6]LH-RH appears to impair both the growth and the hormone-secreting capacity of the implanted tumor. This is analogous to the observations by Kraenzlin *et al.* for the Bauer analog in a patient with vasoactive intestinal peptide (VIP)-producing tumor (16).

The efficacy of [D-Trp^6]LH-RH in inhibiting tumor growth seemed to be dependent on the interval between the transplantation of tumor cells and the initiation of treatment. The greatest reduction in tumor growth was obtained when the treatment was started early. Similarly, it could be surmised that longer periods of treatment with the peptide might produce a greater inhibition of tumor growth. This assumption remains to be proven experimentally.

The mechanism(s) by which [D-Trp^6]LH-RH and other analogs of LH-RH inhibited the growth of the estrogen-dependent ACTH/PRL-producing 7315a rat pituitary tumor has been linked to the suppression of sex steroids levels (6, 7). In the present study with GH_3 pituitary tumor, we have also found that the levels of sex steroids were greatly depressed

Table 4. Effect of administration of analogs of LH-RH for 4 wk on tumor volume and incidence and tumor and ovarian weights in female Wistar/Furth rats bearing pituitary GH_3 tumor

Treatment	Tumor volume, mm^3		Increment in tumor volume,† mm^3	Tumor weight, g	Ovarian weight, mg
	Initial	Final*			
		Experiment II			
Control	445 ± 133	9005 ± 2390	8277 ± 2113	8.6 ± 2	53 ± 6
[D-Trp^6]LH-RH microcapsules	397 ± 93	4326 ± 1772	3380 ± 1501‡	3.6 ± 1§	24 ± 2¶
LH-RH antagonist‖	390 ± 172	5408 ± 2860	5142 ± 2704	6.0 ± 3	25 ± 2¶
		Experiment III			
Control	912 ± 476	7159 ± 3505	6067 ± 3057	6.6 ± 3.2	78 ± 2
[D-Trp^6]LH-RH microcapsules	872 ± 414	2863 ± 983**	1991 ± 758**	2.0 ± 0.7**	29 ± 2**

In experiment I, treatment with the analogs was started 6 wk after inoculation of the tumor cells to the rats. In experiment II, treatment with the microcapsules was initiated 13 wk after inoculation of the tumor cells.
*Animals without a tumor by the end of the experiment were not included.
†All animals, whether with or without tumor, were included in the calculation of this parameter.
‡$P < 0.05$ vs. control by Student's t test.
§$P < 0.05$ vs. control by Student's t test.
¶$P < 0.01$ vs. control by Student't t test.
‖*N*-Ac[D-Phe(4Cl)^{1,2}, D-Trp^3, D-Arg^6, D-Ala^{10}]LH-RH at 50 μg b.i.d.
**$P < 0.01$ vs. control by Wilcoxon test.

Table 5. Effect of administration of analogs of LH-RH for 4 wk on pituitary and serum levels of PRL, GH, and LH in female Wistar/Furth rats bearing pituitary tumor GH_3

	PRL in			GH in			LH in		
	Serum			Serum			Serum		
Treatment*	Pre-,† ng/ml	Post-,† ng/ml	Pituitary, ng/mg	Pre-,† ng/ml	Post-,† ng/ml	Pituitary, μg/mg	Pre-,† pg/ml	Post-,† pg/ml	Pituitary, ng/mg
Control	29 ± 8	554 ± 187	560 ± 34	174 ± 77	9005 ± 3948	35 ± 7	472 ± 67	453 ± 45	959 ± 241
[D-Trp^6]LH-RH microcapsules	14 ± 4	138 ± 67‡	337 ± 53§	132 ± 33	4913 ± 3417	62 ± 11‡	408 ± 54	438 ± 49	37 ± 5§
LH-RH antagonist	31 ± 12	329 ± 242	450 ± 59	168 ± 63	8168 ± 4311	45 ± 8	537 ± 74	229 ± 24§	562 ± 84

*Treatment with the analogs was started 6 wk after inoculation with tumor cells.
†Pre-, hormone levels in serum before initiation of the treatment; Post-, hormone levels in serum after 4 wk of treatment with the analogs.
‡$P < 0.05$ vs. control by Student's t test.
§$P < 0.005$ vs. control by Student's t test.

after treatment with [D-Trp^6]LH-RH. However, the LH-RH antagonist used in this study reduced ovarian weights to the same degree as did [D-Trp^6]LH-RH administration but did not substantially affect tumor growth. Thus, a relationship between reduced ovarian weights (and function) and inhibition of tumor growth seems difficult to envision for the GH_3 pituitary tumor, which suggests that other factors may be involved. Moreover, GH_3 tumor was stated to be estrogen independent (17).

Pituitary LH levels were always decreased by [D-Trp^6]LH-RH administration, regardless of the injection regime used. However, serum LH levels were greatly increased after daily subcutaneous injections of 25 μg b.i.d. of this analog, while [D-Trp^6]LH-RH microcapsules did not modify serum levels of LH. This effect on serum LH levels has been observed with even lower doses of the peptide given as s.c. injections twice a day (unpublished observations). The microcapsule formulation, by maintaining a continuous therapeutic blood level of [D-Trp^6]LH-RH, may desensitize the pituitary gland more effectively to the agonist, whereas twice-a-day injections of the agonist still allow the gland to respond to the stimulatory effect of the peptide with the release of immunoreactive LH (11). A greater efficacy of microcapsules as compared with daily injections also was established previously in our study with rat prostate tumors (11). Our clinical results also attest to the high efficacy of microcapsules (11). In addition, the immunoreactive LH produced after chronic stimulation with LH-RH agonists was reported to have decreased biological activity (18, 19). In any case, ovarian suppression after chronic [D-Trp^6]LH-RH administration may occur in the presence of high immunoreactive serum LH levels (18, 19). However, the antitumoral activity of [D-Trp^6]LH-RH could be related in part to a direct action on this agonist on the pituitary tumor cells.

The GH_3 pituitary cells have been reported to possess receptors for somatostatin (20) and to be unresponsive to GH-RH stimulation (21). Interestingly, the somatostatin analogs used in this study did not inhibit tumor growth, but rather enhanced it under our conditions. Serum PRL and GH levels were elevated over control values in animals treated with the Veber cyclic hexapeptide or the somatostatin octapeptide related to the Bauer analog. Only pituitary GH levels were reduced by the somatostatin analogs, suggesting that the peptides impaired the activity of the normal somatotrophic cell without affecting the growth of GH_3 cells. On the other hand, we have shown previously that the D-5-methoxytryptophan-8 analog of somatostatin inhibited the growth of the pituitary tumor 7315a (7). Furthermore, the original Bauer octapeptide (13) was recently reported to decrease high GH levels and to inhibit tumor growth in a patient with a GH-RH-secreting tumor of the gut (22). Thus, it appears that some, but not all, pituitary tumors can be affected by administration of somatostatin analogs.

In conclusion, administration of [D-Trp^6]LH-RH inhibits the growth of the GH- and PRL-secreting GH_3 pituitary tumor. The exact mechanism(s) through which this effect is exerted are not clear, but the suppression of ovarian functions or a direct action of the analog might be partially involved in this inhibition. The fact that [D-Trp^6]LH-RH can inhibit prolactin levels in ovariectomized animals treated with haloperidol suggests that the suppression of the pituitary–ovarian axis is not necessary for this response (23). Our results indicate a possible application of [D-Trp^6]LH-RH for the treatment of patients with pituitary tumors who fail to respond to conventional therapy.

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