Vol. 81, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1978 Pages 382-390

POTENT AGONIST AND ANTAGONIST ANALOGUES OF LULIBERIN CONTAINING AN AZAGLYCINE RESIDUE IN POSITION 10

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Received January 30,1978

SUMMARY

Potent agonist and antagonist analogues of luliberin containing an azaglycine residue in position 10 were synthesised and tested in androgen-sterilised constant-oestrus rats. The agonist, [D-Ser(Bu^t)^6, Azgly^{10}]-luliberin, induced ovulation at a dose of 6ng/rat i.v., 10µg/rat p.o. and was at least five times as potent as [D-Ser(Bu^t)^6, des-Gly-NH$_2^{10}$, Pro-ethylamide^9]-luliberin. [D-Ser(Bu^t)^6, Azgly^{10}]-luliberin (1µg/rat) also prevented HCG-induced increases in ovarian and uterine weight in immature rats and was a highly potent antitumour agent when given to rats bearing DMBA-induced mammary tumours. The antagonist, [D-Phe^2, D-Phe^6, Azgly^{10}]-luliberin at a dose of 15µg/rat completely inhibited ovulation induced by luliberin (0.5µg/rat), whereas [D-Phe^2, D-Phe^6]-luliberin lost activity below 125µg/rat.

The possibility that an α-aza-amino-acid substitution in a biologically active peptide may generate analogues with either increased potency and longer duration of action or with antagonist activity (1) led to the synthesis of several α-aza-analogues of luliberin (luteinizing hormone-releasing hormone). Such analogues may be useful in man as contraceptive (2,3) and antitumour agents (4,5)and for the treatment of various infertility states (6), in farm animals they may be used to improve fertility (6).

Preliminary work showed that substitution of either azaglycine or azaalanine in position 6 and 10 of luliberin gave analogues which retained biological activity (7). The analogues, [Azgly^6]-, [Azala^6]-, and [Azgly^{10}]-luliberin were as potent as luliberin in inducing ovulation in androgen-

0006-291X/78/0812-0382$01,00/0

sterilised, constant-oestrus (AFO) rats, but appeared less active in releasing FSH and LH in immature male rats (7).

The synthesis has now been completed of several α-aza-analogues of luliberin combining an aza-change in position 6,9 or 10 with either des-histidine modification in position 2 or D-amino-acid replacement in position 2 and/or 6. [D-Phe^2, D-Phe^6]-luliberin (8) and [D-Ser(Bu^t)^6, des-Gly-NH$_2^{10}$, Pro-ethylamide^9]-luliberin (9) were also prepared for comparison. The biological activity of the more potent aza-analogues is now described.

SYNTHESIS

The synthetic procedure used for the preparation of [D-Phe^2, D-Phe^6, Azgly^{10}]-, [D-Ser(Bu^t)^6, Azgly^{10}]-, [D-Phe^2, D-Phe^6]-, and [D-Ser(Bu^t)^6, des-Gly-NH$_2^{10}$, Pro-ethylamide^9]-luliberin is described in Scheme 1. Three peptide fragments, pGlu-A-Trp-Ser-NHNH$_2$ (A = His or D-Phe), N-Z-O-BZL-Tyr-B-Leu-NHNH$_2$ (B = D-Phe or O-Bu^t-D-Ser), and N$^\alpha$-Boc-N$^\omega$-nitro-Arg-Pro-C (C = Azgly, -NHC$_2$H$_5$) were prepared in a stepwise manner by using dicyclohexylcarbodiimide-hydroxybenzotriazole as coupling reagent. The t-butoxycarbonyl protecting group was cleaved by treatment with HCl in ethylacetate, and benzyloxycarbonyl, benzyl and nitro groups were cleaved by hydrogenolysis. The analogues were purified by gel filtration on Sephadex LH-20 in dimethylformamide and by partition column chromatography on Sephadex G-25 using either n-butanol-acetic acid-water (4:1:5) or n-butanol-acetic acid-water-pyridine (5:1:5:1) solvent system. They were characterised by thin layer chromatography in several solvent systems, amino-acid analysis after hydrolysis in 6 N HCl containing 1% phenol at 110°C, and paper electrophoresis at pH6.5 and 2.1.

BIOLOGICAL TEST SYSTEMS

Induction of ovulation.— Agonist activity was determined in AFO rats. In these animals a single i.v. injection of luliberin induces ovulation and a vaginal smear change from cornified to predominantly leucocytic. The ana-

logues were either injected (i.v.; s.c.) or given orally and daily vaginal smears were taken and examined. Three days after dosing the rats were killed and the ovaries examined for the presence of corpora lutea.

Inhibition of ovulation.— Antagonist activity was assessed by the ability of the analogues to prevent ovulation induced by luliberin (0.5μg/rat) in AFO rats. The analogues were injected either s.c. or i.v. simultaneously with luliberin (0.5μg/rat) and the ovaries were examined three days later.

Inhibition of human chorionic gonadotrophin activity.— Twenty-two day old female rats, in groups of 5, were dosed s.c. twice daily with either HCG alone, or combined with a luliberin analogue. The total dose of HCG, given in 6 equal parts, was 50 USP units/rat. Five animals given saline served as untreated controls. On the fourth day of the experiment the rats were weighed and killed and their ovaries and uteri were removed, blotted dry and weighed.

Antitumour activity.— Female rats were dosed orally twice on either day 43 or 44 of age with 9,10, Dimethyl-1,2, benzanthracene (DMBA; 20μg/rat). After 100 days the surviving rats were examined and the tumour sizes were measured by calipers along the longest axis and at right angles to it. The two values obtained were multiplied together to obtain an assessment of tumour area. The rats were then allocated so as to give approximately equal tumour numbers and sizes in control and treated groups. The animals were then dosed for 28 days either with saline or the agonist analogue (5μg/rat twice daily on weekdays and 5μg/rat daily on Saturdays and Sundays). Tumour numbers and sizes were re-assessed weekly.

RESULTS

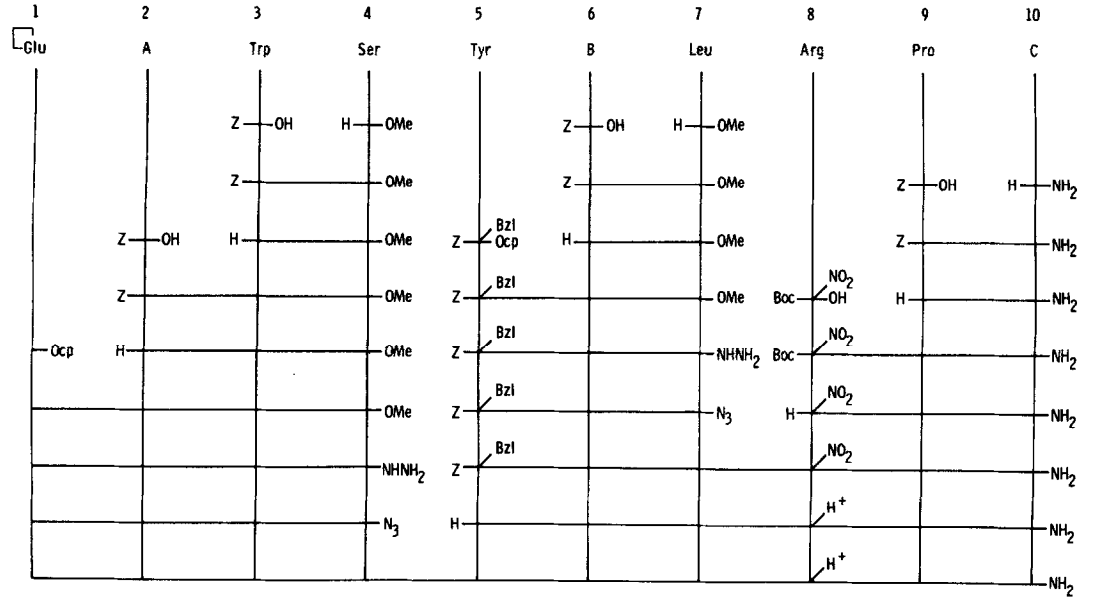
a) Agonists

The potency of the agonists in inducing ovulation in AFO rats is recorded in Table 1. [D-Ser(Bu^t)^6, $Azgly^{10}$]-luliberin induced ovulation at a dose of 0.0063μg/rat when injected i.v. and when given orally was fully effective at 10μg/rat and partially effective at 2.5μg/rat. The related non-aza compound [D-Ser(Bu^t)^6, des-Gly-NH_2^{10}, Pro-ethylamide^9]-luliberin, was fully active intravenously at 0.1μg/rat and partially active at 0.01μg/rat. In this case, therefore, the aza-substitution appears to have increased the biological activity at least 5-fold.

At very much higher doses both [D-Ser(Bu^t)^6, $Azgly^{10}$]-and [D-Ser(Bu^t)^6, des-Gly-NH_2^{10}, Pro-ethylamide^9]-luliberin cause an inhibition of the increase in ovarian and uterine weights induced by HCG (Table 2), the former again proving significantly ($P < 0.05$) more potent.

Table 1. Effect of luliberin analogues on ovulation in androgen-sterilised constant-oestrus rats.

Compound	Dose(μg/rat)		No. Ovulating/No. treated
	Analogue	Luliberin	
Luliberin		0.5 (i.v.)	3/3
		0.25 (i.v.)	3/4
		0.10 (i.v.)	0/4
[D-Ser(Bu^t)^6, Azgly^{10}]-luliberin	0.5 (i.v.)		3/3
	0.0062 (i.v.)		3/3
	0.0031 (i.v.)		0/3
	10 (p.o.)		3/3
	1.0 (p.o.)		3/6
[D-Ser(Bu^t)^6,des-Gly-NH_2^{10},Pro-ethylamide^9]-luliberin	0.5 (i.v.)		3/3
	0.1 (i.v.)		3/3
	0.01 (i.v.)		1/3
[D-Phe^2, D-Phe^6-Azgly^{10}]-luliberin	250 (i.v.)		0/3
	15.6 (i.v. or s.c.)	0.5	0/3
	7.8 (i.v. or s.c.)	0.5	1/3
[D-Phe^2, D-Phe^6]-luliberin	125 (i.v.)	0.5	0/3
	62.5 (i.v.)	0.5	1/3



Scheme 1. Synthesis of luliberin analogues. A= His, D-Phe; B= D-Phe, D-Ser(Bu^t); C= Azgly-NH_2, -NHC_2H_5.

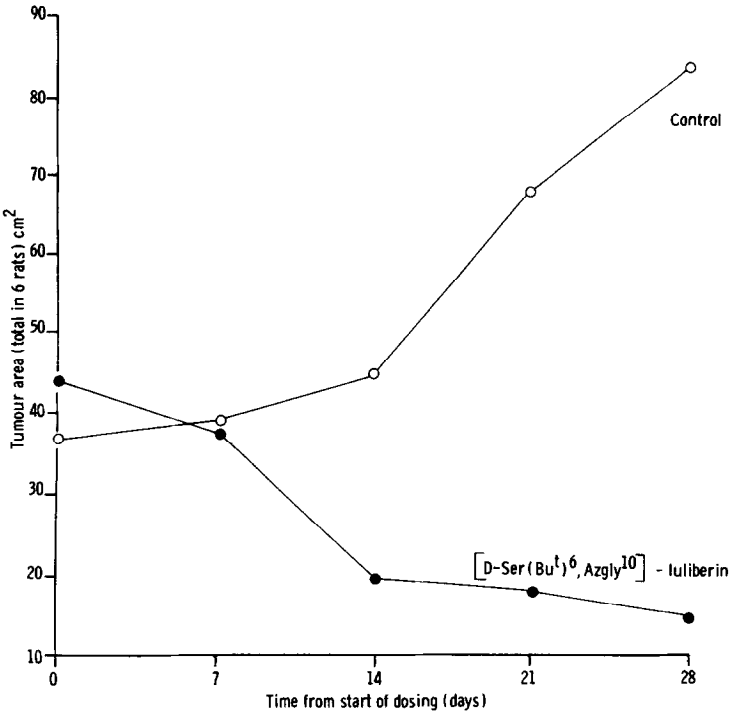


Figure 1. Effect of [D-Ser(Bu^t)^6, $Azgly^{10}$]-luliberin on DMBA-induced rat mammary tumours.

The paradoxical inhibiting effects of high doses of luliberin analogues on ovarian function are also illustrated by the response of DMBA-induced rat mammary tumours to treatment with [D-Ser(Bu^t)^6, $Azgly^{10}$)-luliberin (Figure 1). In untreated control animals tumour size increased markedly and a number of new tumours appeared. In the treated animals some tumours disappeared completely and the remainder showed a highly significant regression. After one month the total tumour area in 6 treated rats was about 14.5 cm^2 whereas in control animals the area increased to around 83 cm^2.

b) Antagonists

[D-Phe^2, D-Phe^6, $Azgly^{10}$]-luliberin, at a dose of 15.6µg/rat completely blocked ovulation induced in AFO rats by 0.5µg luliberin; a partial response was seen at 7.8µg/rat (Table 1). Comparison with the results obtained for

Table 2. Effect of luliberin analogues on HCG-stimulated ovarian and uterine growth. Significantly different from saline controls, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; significantly different from HCG controls, + $P < 0.05$, ++ $P < 0.01$, +++ $P < 0.001$.

Treatment	Dose/rat		Organ Weight. Mean ± S.E.M. (mg/100g Body Weight)	
	Peptide (μg)	HCG (U.S.P.)	Ovaries	Uterus
Experiment 1.				
Saline Control	0	0	49.0 ± 5.4	60.9 ± 5.4
HCG Control	0	50	81.5 ± 7.5**	244.2 ± 13.3 ***
[D-Ser(Bu^t)^6, $Azgly^{10}$]-luliberin	5	50	47.6 ± 6.2++	71.7 ± 16.9+++
[D-Ser(Bu^t)^6,des-Gly-NH_2^{10}, Pro-ethylamide^9]-luliberin	5	50	49.4 ± 3.9++	70.0 ± 7.3+++
[D-Phe^2,D-Phe^6, $Azgly^{10}$]-luliberin	5	50	109.0 ± 14.3**	220.6 ± 10.9***
Experiment 2.				
Saline Control	0	0	44.7 ± 3.2++	45.8 ± 3.0+++
HCG Control	0	50	77.3 ± 6.9**	248.0 ± 8.3***
[D-Ser(Bu^t)^6,$Azgly^{10}$]-luliberin	1	50	49.5 ± 4.5++	112.0 ± 16.1 ** +++
[D-Ser(Bu^t)^6,des-Gly-NH_2^{10}, Pro-ethylamide^9]-luliberin	1	50	62.2 ± 1.2***	159.3 ± 15.0 *** +++

[D-Phe^2, D-Phe^6]-luliberin shows that the azaglycine containing analogue is about 8 times as potent. When given orally (500µg/rat), either simultaneously or 30 or 60 minutes before an i.v. injection of 0.5µg luliberin/rat, [D-Phe^2, D-Phe^6, Azgly^{10}]-luliberin was ineffective in preventing ovulation. The analogue did not show any agonist activity up to a dose of 250µg/rat i.v.; higher doses were not given because of the poor solubility of the compound in saline.

Finally [D-Phe^2, D-Phe^6, Azgly^{10}]-luliberin failed to inhibit HCG-induced increases in uterine and ovarian weight (Table 2) and was ineffective in reducing number and size of DMBA-induced mammary tumours. Indeed it appeared to cause a further increase in ovarian weight (Table 2) when given with HCG and seemed to accelerate tumour growth.

DISCUSSION

Aza-amino-acid substitution in biologically active peptides has previously been attempted in the hope of increasing biological activity but the approach has met with little success. A small increase in potency was observed in the case of [5-azasparagine]-eledoisin (4-11)-octapeptide (10) and [Azgly^9]-oxytocin (11), but most of the other analogues produced were considerably less active. The analogues of luliberin described here are the first examples where the biological potency (agonist and antagonist) has been significantly increased as a result of aza-substitution. It may be speculated that the azaglycine residue in position 10 along with a D-amino-acid residue in position 6 is able to change the overall conformation of the molecule and that the resulting analogues are able to interact more strongly with the receptor sites. Alternatively the increased activity may be due to increased stability of the analogues to enzymic degradation. Although positions 6 and 10 in luliberin have been shown to be susceptible to enzymic action (12), the significance of the metabolic stability in this case should not be over

emphasised because ethylamide substitution is also likely to have such an effect. However [D-Ser(Bu^t)^6, des-Gly-NH_2^{10}, Pro-ethylamide^9]-luliberin is undeniably less potent than [D-Ser(Bu^t)^6, Azgly^{10}]-luliberin. Aza-substitution is also likely to influence the absorption and distribution properties of these analogues, but, at present, it is not possible to assess the importance of these factors.

The results presented confirm earlier reports of the paradoxical inhibitory effects of potent luliberin agonists on HCG-induced gonadal and uterine growth (13) and on DMBA-induced mammary tumours(5,6), but they fail to elucidate the mode of action of these compounds. The antagonist [D-Phe^2, D-Phe^6, Azgly^{10}]-luliberin has been shown to enhance HCG-induced increases in ovarian and uterine weights. This observation taken together with the apparent stimulation of DMBA-induced mammary tumours urges caution in the use of luliberin antagonists in malignant disease. However, a much stronger case can now be made for the study of luliberin agonists as therapeutic agents in tumours of the gonads, breast and reproductive tract.

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