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NOVEL ANTITUMOR PEPTIDE HORMONES AND THEIR EFFECT ON SIGNAL TRANSDUCTION

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Summary—A series of novel gonadotropin releasing hormone (GnRH) and Somatostatin analogs have been developed in our laboratory and were screened for antiproliferative and signal transduction inhibitory effect. Our GnRH analog Folligen, had significant antitumor activity on DMBA induced mammary carcinomas in rats without blocking ovarian functions. The direct effect of Folligen and Buserelin has been compared on the human breast cancer cell line MDA-MB-231. Folligen was found to be more effective in inhibiting cell proliferation and significant differences were found in the signal transduction pathways activated by these analogs. Our novel Somatostatin analogs were screened for tyrosine kinase inhibition and for antiproliferative effect on human colon tumor cells and for growth hormone (GH) release inhibition *in vitro* and *in vivo*. The analog TT-2-50 was significantly more active inhibiting GH release in superfused rat pituitary cells and *in vivo* than native Somatostatin and it strongly inhibited tyrosine kinase and proliferation while it stimulated protein kinase C activity.

INTRODUCTION

Tumor evolution *in vivo* as a multistep process requires that the normally interdependent systems controlling proliferation and differentiation are uncoupled. According to our present knowledge, the regulation of cellular growth is controlled by a complex relationship between pieces of genetic information represented partly by the cellular proto-oncogenes and a series of growth factors and inhibitory factors which provide the fine tuning of relative proliferation rates necessary to coordinate the growth of cells. During tumorous transformation the conversion of proto-oncogenes to activated oncogenes (induced by radiation, chemical carcinogenes, disturbed homeostasis and/or paracrine–endocrine network) include genetic alterations which result in altered levels of expression of the normal protein product, or in normal or altered levels of expression of an abnormal protein. The close relationship between oncogenes and growth factors has been demonstrated by the finding that many oncogenes encode for proteins which are either growth factors or growth factor receptors themselves or they may interact with the growth factor–signal transduction pathways. Two major pathways are involved in the action of growth factors: activation of tyrosine kinases and activation of the phospholipid turnover – protein kinase C pathway. The various steps of these separate signal pathways are potentially the sites of action of oncogenes [1].

Intercellular communication was found to be the critical step in maintaining the dedicated and differentiated functions of cells in a multicellular organism. Failure in this communication network can lead to various malfunctions including tumorous growth. Carcinogenesis can be conceived as a distortion in intercellular signalling and in intracellular signal transduction which can be originated for example from false messages by hormones or growth factors or false signal transduction generated by oncogenes. Since hormones or hormone like substances can send false messages for tumorous growth, it was plausible to consider that other hormones or hormone like substances of the same network can interfere with this communication system and might switch off such mechanisms.

The antitumor activity of certain peptide hormones has been in the center of interest of several research groups in the last years. Two kinds of compounds emerged from these studies: superactive gonadotropin releasing

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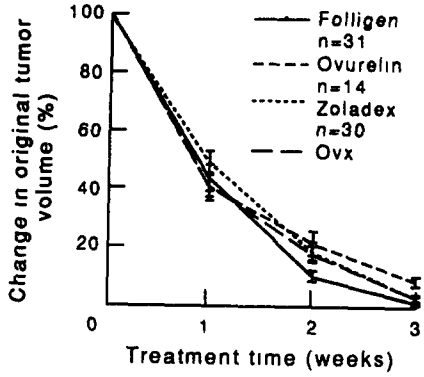

Fig. 1. Effect of Folligen, Ovurelin-C and Zoladex on DMBA induced mammary carcinoma in rats. Rats were treated with 5 μg hormone twice daily for 3 weeks as it is described in Materials and Methods. Control groups had ovariectomy or received no treatment. Values are mean ± SEM $n = 15$ where is not stated otherwise.

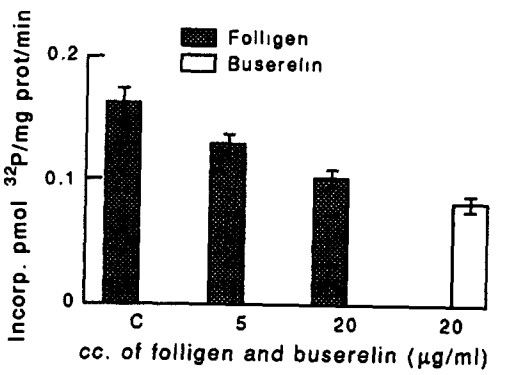

Fig. 3. Effect of Folligen and Buserelin on tyrosine kinase activity of the MDA-MB-231 cells. Cells were incubated for 24 h with or without hormones then harvested and homogenized. Tyrosine kinase activity was measured as described previously, using synthetic peptide substrate. Values are mean ± SEM, $n = 6$.

hormone (GnRH) analogs and Somatostatin analogs. At present, some superactive GnRH analogs and a Somatostatin analog are already used in clinical practice for treating breast, ovarian and prostate cancer [2]. We have developed and patented a series of novel GnRH and Somatostatin analogs and characterized them for antitumor activity.

The use of superactive GnRH analogs for treating various hormone sensitive tumors has been widely accepted recently and the results are very promising [3]. The application of superactive GnRH analogs in the case of neoplasms, benign as well as malignant, include treatment of prostate and breast cancer, pancreatic cancer, pituitary tumors, ovarian cancer and neoplasms of the female genital tract, and other hormone dependent tumors.

Regarding the mechanism of action of these superactive GnRH analogs, it is known that an acute injection of superactive agonists of GnRH induces a marked and prolonged release of LH and FSH, while chronic administration produces dramatic inhibitory effects through a process of "down-regulation" of pituitary membrane receptors for GnRH, desensitization of the pituitary gonadotrophs and reduction in gonadal receptors for LH and FSH. This castration like sex steroid deprivation and the elimination of the stimulatory effects of estrogen or testosterone, is the basis for the application of superactive GnRH analogs in the treatment of hormone dependent tumors. There is no known GnRH agonist at present which exerts antitumor activity via a different mechanism of action [4]. Our novel GnRH analog Folligen opens new perspectives in this respect.

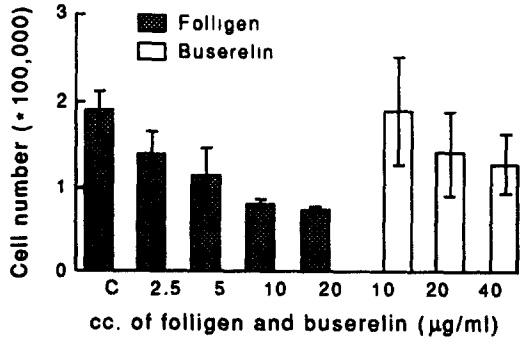

Fig. 2. Effect of Folligen and Buserelin on the growth of MDA-MB-231 human breast carcinoma cells. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and were incubated with or without hormones for 24 h. The cells were washed with phosphate-buffered saline; then 0.25% trypsin was added to detach them. After resuspending, the cells were stained with trypan blue and counted in a Burker chamber. Values are mean ± SEM, $n = 6$.

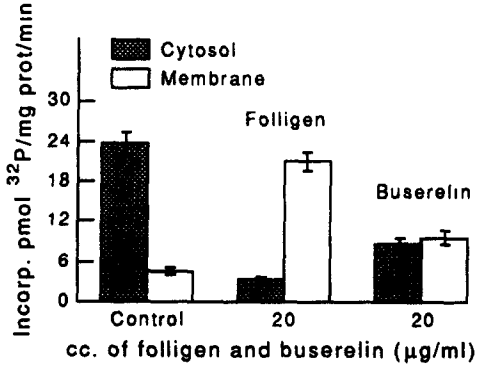

Fig. 4. Effect of Folligen and Buserelin on the activity of protein kinase C in the MDA-MB-231 cell line. Cells were incubated for 24 h with or without hormones then harvested and homogenized. Protein kinase C activity of the cytosol and the membrane fraction was determined as described previously. Values are mean ± SEM, $n = 6$.

Table 1 Tyrosine kinase activity of Somatostatin and its analogs on HT 29 human colon carcinoma cell line

Code No	Dose (μg)	Inhibition (%)
SS-14	10	48
	30	58
TT 2-20	1	35
	10	22
TT 2-50	10	50
	30	90
MK 1-43	10	30
	30	45
AH 25	10	17
	30	23
MK 1-42	10	Inactive
	30	Inactive

The other family of peptide hormones, the Somatostatin analogs, should not be considered as just peptide hormones, but as general growth inhibitory peptides and regulators of signal transduction. Somatostatin is a native polypeptide hormone which inhibits the release of growth hormone, glucagon and insulin and has demonstrated antitumor activity on certain hormone dependent tumors, especially gastrointestinal and gonadal tumors and breast cancer. It was also demonstrated recently that Somatostatin analogs can stimulate phosphatase activity in tumor cells thereby inhibiting signal transduction and tumor growth [5].

Several Somatostatin agonists have been developed and patented during the last few years in order to get a potent antitumor agent. We have developed and patented very recently some novel Somatostatin analogs, which were found to have significant antitumor activity in various test systems. We screened and selected our Somatostatin analogs on the bases of inhibition of tyrosine kinases and proliferation in tumor cells.

MATERIALS AND METHODS

Folligen [(D-Phe^6,Gln^8,desGly^{10})-GnRH-ethylamide] and the Somatostatin analogs were synthesized, purified and characterized in our laboratory as has been described previously [6]. The purity of the peptide preparation was >99% as assessed by thin layer chromatography, amino acid analysis and two validated HPLC methods. The structure of the selected synthetic peptides has also been confirmed by mass spectrometry. Ovurelin-C [D-p-Cl-Phe^6)-GnRH-ethylamide] has been obtained from Reanal Fine Chemicals (Budapest), Zoladex (ICI 118630) [(DSer(^{t}Bu)^6, Azgly^{10})-GnRH], from ICI Pharmaceuticals and Buserelin [(DSer(^{t}Bu)^6,desGly^{10}-GnRH-ethylamide] from Hoechst.

In vivo experiments with rats suffering from DMBA induced mammary carcinomas were carried out according to published producers [7]. In a representative experiment, fifteen 20-week-old female Sprague–Dawley rats (weighing approx. 200 g) were treated intramuscularly (i.m.) with Folligen, Zoladex, or Ovurelin-C (10 μg/day, twice daily for 3 weeks). The weight and characteristic dimensions of the tumors were measured directly before the treatment and then 1, 2 and 3 weeks after beginning the treatment.

In vitro experiments with MDA-MB-231 human breast carcinoma cells and HT-29 human colon carcinoma cells—including measurement of [^{3}H]thymidine incorporation, cell proliferation, tyrosine kinase and protein kinase C assay—have been carried out as described previously [8, 9].

The growth hormone release inhibitory activity of the analogs was tested *in vitro* for their ability to inhibit the growth hormone releasing hormone (GHRH) induced release of radioimmunoassayable GH from superfused rat anterior pituitary cells. The inhibitory effect of Somatostatin (1–14) served as control to evaluate the potency of a given analog.

For *in vivo* testing rats were anaesthetized and morphine was given to increase the basal GH level. Somatostatin or the analogs were administered subcutaneously. 15 min later a blood sample was taken from the jugular vein. GH content of the sera was analyzed by RIA. Potencies were calculated by four point assay and expressed as the percentage of Somatostatin activity [10].

RESULTS AND DISCUSSION

We have developed a series of novel GnRH derivatives—derived from species specific GnRH sequences. Two of them were found to be very potent in stimulating not only ovulation but also follicular maturation and spermatogenesis in various kinds of fishes and mammals. One of these analogs was selected for further studies and named: Folligen [6]. The fact that Folligen induces follicular maturation and ovulation in cases where mammalian GnRH analogs have not worked raised the possibility that Folligen acts via a special mechanism of action which may involve a direct gonadal action.

Table 2 *In vitro* and *in vivo* effect of Somatostatin analog (TT 2-50) on the GH-RH induced release of GH compared to Somatostatin (SS-14)

	In vitro inhibition		*In vivo* inhibition			
Analog	Dose (M)	Inhibition of GH release (%)[a]	n[b]	Dose (μg/100 gBW)	GH level[c]	Potency[d]
Saline			9	—	86 36 + 2 17	
SS-14	10^{-7}	100	9	0 05	58 78 + 3.36	100
	10^{-8}	50	9	1.00	31 76 + 1 56	
TT 2-50	10^{-8}	100	9	0 01	53 80 + 2 93	580
			9	0 20	41 90 + 4.13	(180–1964)

[a]Inhibition of GHRH induced release of radioimmunoassayable GH from rat anterior pituitary cells
[b]Number of animals
[c]GH levels are expressed as % of initial serum GH levels
[d]Potency was determined by four-point assay The 95% confidence limits are given in parentheses

We investigated the antitumor activity of Folligen both *in vitro* and *in vivo*. The *in vivo* effect of Folligen has been investigated on DMBA induced mammary carcinomas in rats by the Tenovus Cancer Research Institute. It was found that Folligen had the same antitumor activity as the widely used superactive GnRH analog, ICI's Zoladex, but Folligen did not block ovarian functions. Folligen caused an almost 100% tumor remission during a 3 week daily treatment (Fig. 1). Although the circulating level of estradiol decreased with Folligen treatment the progesterone level increased and the histological picture of the ovaries showed developing follicles and corpora lutea. Since these *in vivo* results strongly suggest that Folligen can have a direct inhibitory effect on breast cancer cells we investigated the effect and the mechanism of action of Folligen on the MDA-MB-231 human breast carcinoma cells. We found that Folligen directly inhibited the proliferation of this human breast cancer cell line and it modulated certain signal transduction pathways which have very important roles in the regulation of cell proliferation. The effect of Folligen on cell proliferation of the MDA-MB–231 cells has been investigated by monitoring cell number over 72 h and its effect was compared to the effect of Buserelin (Fig. 2). We found that both Folligen and Buserelin significantly inhibited proliferation, Folligen being more effective. Regarding the signal transduction pathways involved we found that the tyrosine kinase activity of the MDA-MB-231 cells during a 24 h incubation was strongly inhibited both by Folligen and Buserelin. Buserelin was more active than Folligen in inhibiting tyrosine kinase activity (Fig. 3). The total protein kinase C activity of the cells was not significantly influenced by either of these hormones. When we investigated the distribution of protein kinase C activity in the cytosol and the membrane of the MDA-MB-231 cells following hormonal treatment we found that Folligen caused a strong shift in the enzyme activity from the cytosol to the membrane while Buserelin also caused a shift but to a much smaller extent (Fig. 4). These results clearly demonstrated that both Folligen and Buserelin directly inhibited the proliferation of these human breast carcinoma cells modulating

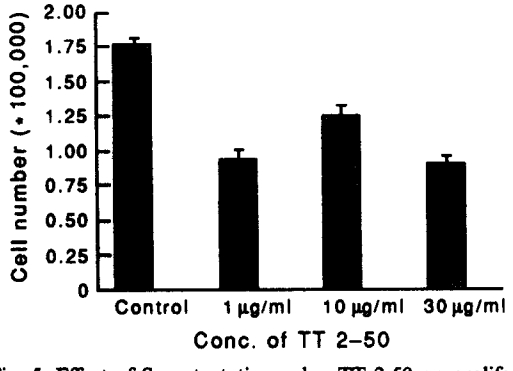

Fig. 5. Effect of Somatostatin analog TT 2-50 on proliferation of the HT 29 human colon tumor cells. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). Cells were incubated with or without hormones for 24 h without serum. After the incubation cells were washed, harvested and counted. Values are mean ± SEM, $n = 6$.

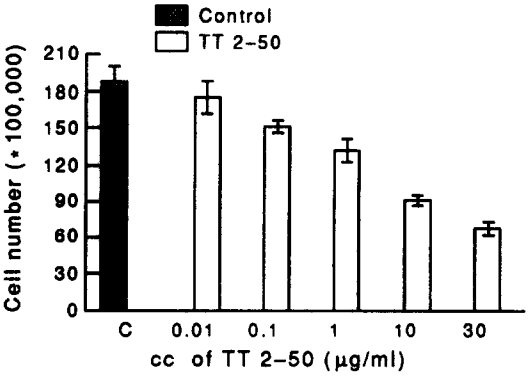

Fig. 6. Effect of Somatostatin analog TT 2-50 on proliferation of SW 620 colon tumor cells. Experimental circumstances are as described in the legend to Fig. 5. Values are mean ± SEM, $n = 6$.

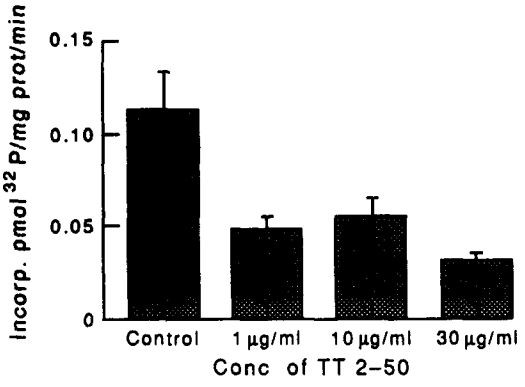

Fig. 7. Effect of Somatostatin analog TT 2-50 on the tyrosine kinase activity of HT 29. Cells were cultured and treated as described in Fig 5. Tyrosine kinase activity was measured as described previously. Values are mean ± SEM, $n = 6$.

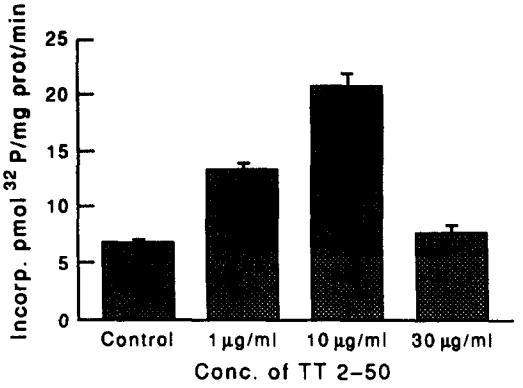

Fig. 8. Effect of Somatostatin analog TT 2-50 on the protein kinase C activity of HT 29. Cells were cultured and treated as described in Fig. 5. Protein kinase C activity was measured as described previously. Values are mean ± SEM, $n = 6$.

various signal transduction pathways probably by using different mechanisms of action.

In our efforts for developing novel signal transduction inhibitory compounds we synthesized a series of new Somatostatin analogs and screened them for tyrosine kinase inhibitory activity and antiproliferative effect in various tumor cell lines. It has been demonstrated earlier that Somatostatin and its analogs activate phosphatases in a pancreatic tumor cell line and it has been suggested that Somatostatin induces a turn off signal for tyrosine kinase in proliferating tumor cells. Several potent Somatostatin analogs have been developed in the last years, with good antitumor activity for certain hormone dependent tumors, but their tyrosine kinase inhibitory activity was not investigated. We investigated the effect of the native Somatostatin SS-14 on the tyrosine kinase and protein kinase C activity and on IP_3 level of the HT-29 human colon tumor cells and found that it strongly inhibits tyrosine kinase activity, while it stimulated protein kinase C and IP_3. We screened our new Somatostatin analogs for tyrosine kinase inhibitory activity (Table 1) and antiproliferative effect in human colon tumor cells as well as for growth hormone release inhibitory activity *in vitro* in superfused rat pituitary cells and *in vivo* in rats.

On the basis of these assays two analogs have been selected for further development; one with strong tyrosine kinase inhibitory, antiproliferative and growth hormone release inhibitory activity (analog TT 2-50) and one with good antitumor activity without growth hormone release inhibitory activity. The effect of the analog TT 2-50 [D-Phe-Cys-Tyr-D-Trp-Lys-Leu-Cys-Thr-NH_2] on growth hormone release (Table 2), proliferation (Figs 5 and 6), tyrosine kinase activity (Fig. 7) and protein kinase C activity (Fig. 8) is shown.

In summary we can conclude that we have developed some new GnRH and Somatostatin analogs with good antitumor activity and demonstrated that they directly modulate signal transduction pathways in human breast and colon tumor cells.

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