Influence on antiproliferative activity of structural modification and conjugation of gonadotropin-releasing hormone (GnRH) analogues

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Abstract. The effect of various GnRH analogues, and their conjugates on proliferation, clonogenicity and cell cycle phase distribution of MCF-7 and Ishikawa human cancer cell lines was studied. GnRH-III, a sea lamprey GnRH analogue reduced cell proliferation by 35% and clonogenicity by 55%. Structural modifications either decreased, or did not alter biological activity. Conjugation of GnRH analogues including MI-1544, MI-1892, and GnRH-III with poly(N-vinylpyrrolidone-co-maleic acid) (P) through a tetrapeptide spacer GFLG(X) substantially increased the inhibitory effect of the GnRH analogues. The conjugate P-X-GnRH-III induced significant accumulation of cells in the G2/M phase; from 8% to 15.6% at 24 h and 9.8% to 15% at 48 h. It was concluded that conjugation of various GnRH analogues substantially enhanced their antiproliferative activity, strongly reduced cell clonogenicity and retarded cell progression through the cell division cycle at the G2/M phase.

Gonadotropin releasing hormone (GnRH) analogues such as buserelin, goserelin, lupron and decapeptyl are used for the therapy of sex hormone-dependent tumours of the breast, prostate and ovaries (Vickery, 1986; Höffken, 1992; Weinbauer & Nieschlag, 1992). These hormone analogues exert their antitumour effects indirectly, by inhibiting the actions of sex hormones via the hypothalamic–pituitary–gonadal axis. There are, however, indications that GnRH analogues suppress the growth of cancer cells *in vitro* (Kleinmann *et al.*, 1994). In extrahypophyseal endocrine-related tumours responsive to GnRH analogues, specific binding sites for GnRH and GnRH receptor mRNA are expressed. The hypothesis is put forward that the effects of GnRH analogues in these cancer cells are mediated via specific GnRH receptors.

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Several new GnRH analogues were synthesized and their *in vitro* antitumour effects were investigated on GnRH receptor-positive cancer cell lines including MDA-MB-231, MCF-7 breast, LNCaP and PC3 prostate and Ishikawa endometrium. In earlier investigations (Mező *et al.*, 1996) minor changes in the structures of GnRH analogues differently affected LH-release in the pituitary and suppression of the proliferation of tumour cells. The human GnRH antagonist MI-1544 (Ac-D-Trp-D-Cpa-D-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-D-Ala-NH₂) had both high endocrine activity (Kovács *et al.*, 1989) and strong antitumour effect (Pályi *et al.*, 1996, 1999). In the *in vitro* LH-releasing activity, the potency of the chicken GnRH antagonist MI-1892 (Ac-D-Trp-D-Cpa-D-Trp-Ser-D-Lys- β -Asp(α -DEA)-Leu-Gln-Pro-D-Ala-NH₂) was below 0.01% of that of GnRH (Mező *et al.*, 1996) while its antitumour activity remained similar to that of MI-1544 (Pályi *et al.*, 1999). A variant, GnRH-III (pGlu-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH₂) from the sea lamprey, *Petromyzon marinus*, caused LH-release only at 1 μ M therefore being a weak agonist. Radioreceptor assay with [3H] GnRH-III showed the presence of high- and low-affinity binding sites in the membrane suspensions of these cells (Vincze *et al.*, 1997; Lovas *et al.*, 1998). GnRH-III significantly suppressed growth of human cancer cells which have GnRH receptors. The inhibitory effect was specific and direct, since the peptide did not have endocrine activity in the concentration range found to be effective in anticancer assays. The antiproliferative effect of the most recently prepared GnRH analogues was 20–40% (Pályi *et al.*, 1996; Mező *et al.*, 1996, 1997; Lovas *et al.*, 1998).

GnRH analogues are subject to degradation by proteolytic enzymes. To avoid their decomposition and to increase stability and biological efficacy, they were coupled with a non-biodegradable copolymer, poly(N-vinylpyrrolidone-co-maleic acid) (P) through a tetrapeptide spacer, GFLG (X). Conjugation of GnRH analogues increased their antiproliferative effect *in vitro*, and antitumour action *in vivo* as described earlier (Pályi *et al.*, 1996; Vincze *et al.*, 1996, 1997; Pályi *et al.*, 1999) and added to their direct effect on cancer cells.

Conjugates of more GnRH analogues were recently synthesized to examine further the contribution of conjugation to enhanced biological activity and the effects of the conjugates on cell cycle progression, especially accumulation of cells in the G2/M phase.

MATERIALS AND METHODS

Peptides

Syntheses of GnRH analogues MI-1544, MI-1892, GnRH-III, SJ-1004, [Lys5, DTrp6]GnRH, [Lys5]GnRH-III, [Lys5,cyclo(Asp6,Lys8)]GnRH-III, [Lys4]GnRH-III, [Phe7]GnRH-III and [D-Ala10]GnRH-III, have been described previously (Kovács *et al.*, 1984; Sower *et al.*, 1993; Mező *et al.*, 1996, 1997; Lovas *et al.*, 1998).

Conjugates

The peptides were conjugated through a Gly-Phe-Leu-Gly (X) spacer to the copolymer poly(N-vinylpyrrolidone-co-maleic acid) (P), previously referred to as NVP MA when details of syntheses were reported (Pató & Tüdös, 1989) to form P-X peptide conjugates. The conjugates contained $\approx$ 10% peptide and had an average molecular weight of 12000.

Cell cultures

The oestrogen receptor (ER)-positive MCF-7 breast cancer cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and the Ishikawa human

endometrium carcinoma cell line from Dr Ch. Marth, Universitäts-Frauenklinik, Innsbruck, Austria. Both cell lines have GnRH receptors. The cells were maintained in Dulbecco's modified Eagle Minimal Essential Medium (GIBCO BRL, Paisley, UK) supplemented with 10% fetal calf serum (GIBCO). Cells were kept at 37°C and subcultured twice each week.

Cell proliferation assay

Proliferation of cells in control and treated cultures were determined as described (Pályi *et al.*, 1996). Briefly, 4×10^4 cells were plated into 50 mm diameter plastic Petri dishes and were treated on the following day with different concentrations of the compounds. Treatment with the GnRH analogues was repeated every other day for 5 days. Cells were then trypsinized and counted in a Neubauer-type haemocytometer. Two or three dishes were used in each group. The number of treated cells was expressed as a percentage of cells in control cultures.

Clonogenicity assay

Cell survival was assessed by colony formation as described previously (Pályi *et al.*, 1996). Briefly, 300 cells were put into 35 mm diameter Petri dishes in 2.5 ml medium. On the following day, different amounts of the compounds were each added to these dishes which were then kept for 8–12 days in a Heraeus CO₂ incubator (Heraeus Instruments GmbH, Hanau, Germany). The cells were rinsed with 0.04 M NaH₂PO₄ – Na₂HPO₄ buffer, pH 7.4, containing 0.15 M NaCl (PBS) and stained with crystal violet. The number of colonies, containing a minimum of 50 cells, was counted using a dissection microscope. The number of colonies in treated cultures was expressed as a percentage of those in untreated control cultures.

Flow cytometry

Cell cycle phase distributions of control and treated cultures were analysed by measurements of relative DNA contents of individual cells using a fluorescence-activated cell sorter. Samples were prepared according to the method of Shapiro (1988). MCF-7 cells were treated with P-X-1544, or with P-X-GnRH-III (50 and 100 µM peptide in each case) for 24 and 48-h periods, released by trypsinization, washed with PBS, fixed in 70% ethanol and stored at –20°C. Before flow cytometry, cells were collected by centrifugation and incubated for 30 min at room temperature in PBS (1 ml) containing 20 g propidium iodide and 100 µM RNase. Measurements were carried out with a FACStar Plus flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA) and with a FACStar Plus Research Program connected with a Doublets Discrimination signal processor. The analytical system was tested using lysed, propidium iodide-stained normal human lymphocytes. Cell cycle analysis was accomplished with Multi-Cycle Software (Phoenix Flow Systems, San Diego, CA, USA).

RESULTS

Structure-antiproliferative/anticlonogenic relationships

The effect of lamprey GnRH-III on the proliferation of MCF-7 cells is shown on Fig. 1. The doubling time of the control cells was about 24 h during the exponential phase of growth, following the initial lag period. Concentrations of GnRH-III between 5 and 50 µM retarded cell proliferation slightly; at 50 µM, the inhibition of growth was only 35%.

In the clonogenic assay (Fig. 2), the effect of GnRH-III was stronger than in the antiproliferation assay, although the cells were treated only once during the experiment.

Figure 1. Growth curves of MCF-7 cells treated with different doses of GnRH-III. Growth of control cells (x) was retarded slightly by exposure to 5 μ M (●), 10 μ M (▲), 30 μ M (▼), or 50 μ M (■) concentrations of GnRH-III. Treatment was repeated on every other day.

Figure 2. Percent survival of MCF-7 cells treated with 50 μ M of GnRH-III (column 1), Lys5-GnRH-III (column 2), DAla10-GnRH-III (column 3), Phe7-GnRH-III (column 4) and Lys5-cyclo(Asp6,Lys8)-GnRH-III (column 5) for 8 days. The Lys5 and DAla10 derivatives destroyed antiproliferative activity, the Phe7 analogue reduced, while the cyclo analogue had no influence on the activity of the GnRH-III analogue.

50 μM GnRH-III reduced colony formation to 55% of the control value. Replacement of His5 with Lys caused a loss of anticolonogenic activity. Replacement of Gly10 with D-Ala also led to nearly complete loss of anticolonogenic activity. Introduction of Phe in place of Trp7 reduced anticolonogenicity to about half that of GnRH-III. [Lys5]GnRH-III had similar anticolonogenic activity to that of GnRH-III.

Effect of conjugation on the biological activity of GnRH analogues

MI-1544 reduced cell survival to 52% at 50 μM . After conjugation, the same peptide reduced cell survival to 40% even at 10 μM and to 2% at 50 μM (Fig. 3a). A similar fall in the number of surviving colonies was found with MI-1892 and its conjugate P-X-1892 (Fig. 3b). P-X-GnRH-III reduced cell survival to 10% at 50 μM (Fig. 3c). SJ-1004 showed moderate anticolonogenic activity (34% inhibition) but this increased to 95% following conjugation (Fig. 3d). Conjugation proved to be less effective in enhancing anticolonogenic activity in the cases of [Lys4]GnRH-III (from 39% to 67% inhibition; Fig. 3e), [Lys5,cyclo(Asp6,Lys8)]GnRH-III (from 40% to 78% inhibition; Fig. 3f).

The enhancement of growth-inhibitory activity by conjugation of GnRH analogues was also shown by the antiproliferative assay (Fig. 4). Proliferation of MCF-7 cells was reduced from 64% to 42% due to conjugation of either MI-1544, or GnRH-III, and from 65% to 33% following conjugation of MI-1892. A bigger reduction (83% to 32%) of cell growth was caused by conjugation of SJ-1004 (Fig. 4).

Effect of GnRH conjugates on cell cycle phases

Table 1 and Fig. 5 show that accumulation of MCF-7 cells in the G2/M phase is considerably increased (from 8.1% to 15.6%) by conjugation of GnRH-III when exposure was for 24 h. The difference was less (from 9.8% to 15%) following 48 h exposure. G2 phase cells accumulated at the expense of G1 and, to a less extent, S phase cells (24 h exposure, Table 1).

DISCUSSION

We have shown that various structural modification of GnRH-III from the sea lamprey either resulted in a decreased antiproliferative and anticolonogenic activity, or did not modify the biological effect of the original GnRH-III molecule. Replacement of a weakly basic and aromatic residue (His5) to a more basic (Lys) residue was accompanied by the loss of activity. Replacement of C-terminal Gly-NH₂ with DAla10 resulted in a complete loss of antiproliferative and anticolonogenic effect. To test the possible interference of the Lys5 side chain with the side chains of Asp6 and Lys8, a steric conformer,

Table 1. Effect of P-X-GnRH-III on phase distribution of MCF-7 cell cycle

Conjugated peptide (μM)	Phase distribution (%)					
	24 h			48 h		
	G1	S	G2/M	G1	S	G2/M
0	51.9	40.0	8.1	56.1	34.1	9.8
50	49.3	39.9	10.7	57.6	31.6	10.8
100	46.7	37.7	15.6	51.6	33.3	15.0

Cells were grown for 24 h and 48 h in the presence or absence of conjugated peptide before flow cytometry as described in the text.

Figure 3. Dose survival curves of MCF-7 cells treated with different GnRH analogues and their conjugates. (a) MI-1544 (●) and P-X-1544 (▲); (b) MI-1892 (●) and P-X-1892 (▲); (c) GnRH-III (●) and P-X-GnRH-III (▲); (d) SJ-1004 (●) and P-X-1004 (▲); (e) Lys4-GnRH-III (●) and P-X-Lys4-GnRH-III (▲); (f) Lys5-cyclo(Asp6,Lys8)-GnRH-III (●) and P-X-Lys5-cyclo(Asp6,Lys8)-GnRH-III (▲). Note the increased activity of conjugates on clonogenicity compared with the GnRH analogues.

Figure 4. Comparison of antiproliferative activity on MCF-7 cells of some GnRH analogues and their conjugates. Columns 1, 3, 5 and 7 (black) represent MI-1544, MI-1892, GnRH-III and SJ-1004, and columns 2, 4, 6 and 8 (grey) represent P-X-1544, P-X-1892, P-X-GnRH-III and P-X-1004 conjugates, respectively. Exposure concentration was 50 μ M; treatment with the GnRH analogues was repeated every other day and with their conjugates occurred only once. Note the increased antiproliferation effect exerted by the conjugates (columns 2, 4, 6, 8).

[Lys5,cyclo(Asp6,Lys8)]GnRH-III was synthesized and tested. By this step, the Lys5 analogue regained antiproliferative activity. The role of the indolyl side chain in the peptide-receptor bonding is supported by the observation that substitution of Trp7 with Phe in GnRH-III caused a 50% decrease of its anticolonogenic effect.

GnRH analogues MI-1544, MI-1892 (Pályi *et al.*, 1996) and GnRH-III (Mező *et al.*, 1997) surpassed the antiproliferative activity of the GnRH agonist buserelin (Blankenstein *et al.*, 1985; Mullen *et al.*, 1991; Höffken, 1992), or the antagonist SB-75 (Segal-Abramson *et al.*, 1992; Kleinman *et al.*, 1993).

Figure 5. Effect of P-X-GnRH-III on phase distribution of MCF-7 cell cycle. a: Control culture b: Culture exposed to 100 μ M conjugate for 24 h.

The antiproliferative and anticlonogenic activity of GnRH analogues was substantially increased by conjugation with P-X. MCF-7 cells treated with P-X-GnRH-III conjugate showed a significant accumulation of G2/M cells. The conjugates, besides their inhibitory effect on signal transduction mechanism, were able to slow down progression of cells from G2 to M phase in 48 h, and this became evident after 5 days in proliferation-retarding effect.

A slight increase (from 24% to 28%) of G2 cells was found using Ishikawa endometrial culture treated with SB-75 GnRH antagonist (Kleinman *et al.*, 1994), while a slight, 5–6% increase of G0/G1 cells in ovarian epithelial tumour cell culture 2774 treated with the GnRH agonist lupron was described (Thompson *et al.*, 1991). Mullen *et al.* (1991) reported a significant accumulation of G0/G1 cells (from 45% to 75%) in a clonal variant of MCF-7 culture treated with the agonist buserelin, while no such changes were observed in three other MCF-7 lines. As the GnRH conjugates inhibit cdc25 phosphatase, a key enzyme for entry of cells into the M phase (Meijer, 1995), it is possible that inhibition of cdc25 phosphatase enzyme contributes to G2 cell accumulation (Pályi *et al.*, 1999).

Coupling of anticancer compounds to various polymers to improve their efficacy commenced in the late 1980s (Duncan *et al.*, 1996). Conjugation enhanced the antitumour activity of the compounds by altering their characteristics including chemical stability, biodistribution and penetration. Here we observed a substantially increased antiproliferative and anticolonogenic activity of all GnRH analogues following conjugation with P-X. The stable, water-soluble and specific GnRH analogue conjugates have strong therapeutic potential.

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