Eur J Cancer Clin Oncol, Vol. 21, No. 12, pp. 1493–1499, 1985.
Printed in Great Britain.

0277–5379/85$3.00+0.00

Direct Inhibitory Effect of a Luteinizing Hormone-releasing Hormone Agonist on MCF–7 Human Breast Cancer Cells*

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Abstract—*The effect of the LHRH agonist Buserelin on the MCF–7 human breast cancer cell line was studied. Cells were cultured in medium containing* 10% *untreated foetal calf serum or* 10% *steroid-depleted serum. In both media the DNA and protein content of cultures kept for* 3–5 days *in the presence of* 80–800 nM *Buserelin and* 1 nM *oestradiol were* 8–27% *lower than those of flasks cultured in the presence of oestradiol alone* ($P < 0.05$). *LHRH itself* (400 nM) *also displayed an antiproliferative effect on the MCF–7 cultures. At an equimolar concentration, the LHRH antagonist ORG* 30093*D abolished the antiproliferative effect of Buserelin. MCF–7 cells did not specifically take up radioiodinated LHRH. Our data are the first to indicate that LHRH analogues may inhibit the growth of MCF–7 cells to a limited extent. The antitumour activity of these compounds* in vivo *may, then, be due to the main pituitary and gonadal effects, resulting in a decrease of the concentration of oestrogen in the circulation and, in addition, a direct effect at the target cell level.*

INTRODUCTION

ANALOGUES of LHRH are currently being evaluated for use in the treatment of advanced breast [1, 2] and prostate cancer [3–5]. When administered over a long period and at a sufficiently high dose, these compounds elicit antifertility effects [6]. Two mechanisms have been suggested which would lead to 'medical gonadectomy'. Firstly, prolonged stimulation with pharmacological doses of LHRH agonists results in exhaustion and/or desensitization of the gonadotropic cells in the pituitary, which in turn leads to a reduced gonadotropin output [7, 8] and, hence, a decreased steroidogenesis. Secondly, direct effects of analogues of LHRH at the gonadal level may also cause a decreased steroidogenesis [9, 10]. In this respect it has been suggested that gonadal cells use a locally synthetized LHRH-like peptide for communication [11]. The direct effects of LHRH-like peptides at the gonadal level have been extensively investigated in the rat. They are thought to be mediated by receptors for LHRH on the membranes of the gonadal cells [9, 12–14]. By analogy with the rat, an LHRH agonist has been reported to inhibit the secretion of progesterone by cultured human granulosa cells [15]. In another study, however, no such effect was observed [16]. Similarly, the presence of receptors for LHRH-like peptides in the human gonads is still a matter of debate [17, 18].

In addition to causing medical gonadectomy, analogues of LHRH have been suggested to interfere with the action of steroid hormones on their target cells [19–21]. LHRH analogues can inhibit oestrogen-induced growth of the rat uterus [21, 22], oestrogen-induced increases in the activity of enzymes associated with uterine cell proliferation [23], androgen-induced growth of rat seminal vesicles and ventral prostate [22] and β-glucuronidase activity in the mouse kidney [20]. From these observations, the working hypothesis has been derived that, apart from impairing ovarian steroidogenesis, LHRH agonists may exert an additional antitumour effect based on interaction with the remaining (adrenal) steroids at the target cell level. The preliminary results of Corbin [19], who reported an inhibitory effect of an LHRH analogue on the growth of mouse mammary tumour cells *in vitro*, support this hypothesis. By contrast, Furr and Nicholson [24] did not observe an antioestrogenic effect of an LHRH analogue in ovariectomized immature rats.

Accepted 1 July 1985.
*This study is supported by the Netherlands Cancer Society (KWF) through grant No. RRTI 83–3. Preliminary data were presented during the Breast Cancer Research Conference, International Association for Breast Cancer Research, Denver, Co, U.S.A. 20–24 March 1983, and the Second International Congress on Hormones and Cancer, Monte Carlo, Monaco, 18–23 September 1983.
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The present study was designed to study the existence of direct antitumour effects of LHRH agonists. In this respect, effects of Buserelin and oestradiol on the human breast cancer cell line MCF-7 and the binding of radioactive LHRH to these cells have been investigated.

MATERIALS AND METHODS

The human breast cancer cell line MCF-7 was obtained from EG&G Mason Research Institute, Worcester, MA, U.S.A., in its 219th passage. Cells were cultured at 37°C in an atmosphere of 5% CO_2 and air in Falcon T-75 culture flasks in RPMI-1640 medium, supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin (Gibco, Grand Island, NY, U.S.A.) and 10 ng/ml insulin (Organon, Oss, The Netherlands). This medium will be referred to as 'fully supplemented medium'. For experiments the cells were trypsinized and seeded in T-25 flasks in fully supplemented medium to allow attachment of the cells to the culture flasks. After one day the medium was changed for the experimental medium, which was changed daily unless indicated otherwise.

Experimental media

Additions to the medium included oestradiol, (Merck, Darmstadt, F.R.G.), tamoxifen (ICI-Farma, Rotterdam, The Netherlands), the LHRH agonist Buserelin (Hoechst-Pharma, Amsterdam, The Netherlands) and the LHRH antagonist [NAc-p-Cl-(D)Phe-1,2, (D)Trp-3, (D)Phe-6, (D)Ala-10]LHRH (ORG 30093D Organon International, Oss, The Netherlands) [25]. These additives were given either alone or in combination at concentrations indicated at the individual experiments. Oestradiol and tamoxifen were added to the medium as concentrated solutions in ethanol. The final concentration of ethanol in the medium never exceeded 0.2% (v/v). Corresponding amounts of ethanol were added to media which did not contain oestradiol or tamoxifen. Working solutions of Buserelin were prepared in Dulbecco's phosphate-buffered saline (Gibco, Grand Island, NY, U.S.A.), which was also added to the media which lacked Buserelin. A concentrated solution of the LHRH antagonist was prepared in 70% ethanol. Working solutions were prepared by dilution with phosphate-buffered saline. In one experiment synthetic LHRH (Relefact, Hoechst AG, Frankfurt-am-Main, F.R.G.) was used. This compound was used directly as supplied. In some experiments FCS was treated for 30 min at room temperature with 0.5% (w/v) Norit and 0.05% dextran T-70. Charcoal was removed by centrifugation for 30 min at 10,000 ***g***. The supernatant is referred to as dextran-coated charcoal-treated FCS (DCCFCS).

Termination of experiments

After the desired culture period the medium was removed and the cells were washed twice with 0.154 M NaCl. Thereafter, cells were dissolved in 1 ml 1 M NaOH at 50°C for 1 hr. The protein concentration of the resulting solution was estimated by the method of Bradford [26] using the kit from Bio-Rad (Richmond, CA, U.S.A.) and human serum albumin (KABI, Stockholm, Sweden) as a standard. DNA was measured with a fluorimetric assay [27]. Diaminobenzoic acid (Merck, Darmstadt, F.R.G.) and herring sperm DNA (Schuchardt, Munich, F.R.G.) were used as reagent and standard respectively. Cell numbers were not counted since it has been described that for the MCF-7 cell line changes in cell number show a consistent correlation with changes in DNA mass [28].

Binding studies with LHRH

Radio-iodinated LHRH was purchased from New England Nuclear (Dreieich, F.R.G.). Radiochemical purity was verified by thin-layer chromatography on cellulose plates (Merck, Darmstadt, F.R.G.) in the system *n*-butanol:water:ethyl acetate = 11:2:1 (v/v/v). In binding studies the cells were washed twice and incubated for 90 min at 4°C with approximately 300,000 cpm of tracer in 10 mM Tris-HCl buffer, pH 7.8, containing 1 mM dithiothreitol and 0.1% bovine serum albumin as described by Loumaye *et al.* [29]. After the incubation, cells were washed twice and dissolved in 1 M NaOH as described above. For comparative purposes, the binding of radioactive LHRH to 25,000 ***g*** membrane preparations [17] of rat pituitary and human breast and ovarian carcinoma tissue was also studied.

In the figures results are given as means ± standard deviation. The number of replicate observations is represented by *n*. Statistical analysis was performed with Wilcoxon's test. Differences were considered to be statistically significant when a P value of less than 0.05 was found.

RESULTS

Effects of Buserelin and tamoxifen

In early experiments we studied the effect of a single administration of Buserelin on the growth of MCF-7 cells. At concentrations of 10 and 100 ng/ml, Buserelin did not affect the protein content of the cells which were cultured for 1-3 days after addition of the drug. In further experiments the medium was changed daily in order to reduce the possibility that putative effects of Buserelin on the cells remain undetected as a result of rapid metabolism (and inactivation) of the peptide. The re-

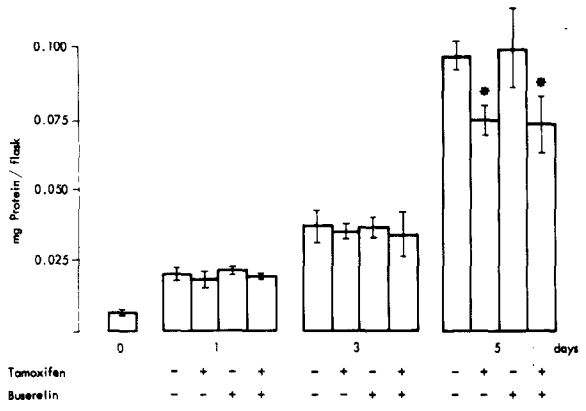

Fig. 1. Protein content of MCF-7 cultures grown in fully supplemented medium at different times during daily administration of 80 nM *Buserelin and/or* 1000 nM *tamoxifen. Data are given as means ± S.D.;* n = 5; *P < 0.05 *vs corresponding control.*

sults in Fig. 1 demonstrate that under the conditions used, i.e. in fully supplemented medium and after daily administration, Buserelin has no effect on the growth of the cells. Tamoxifen, on the other hand, significantly decreased the growth rate of the cells ($P < 0.05$), as evidenced by a lower protein content of the cultures. Buserelin did not alter the response of the cells to tamoxifen.

Comparison of protein and DNA content

To justify the use of the protein content of the cultures for the evaluation of the results of the present experiments, this parameter was compared to the DNA content of the cultures. An excellent correlation was found between these parameters. In 36 sets of data originating from four different experiments a correlation coefficient of 0.863 ($P < 0.001$) was found. The intercept of the regression line [DNA = 0.22 × protein − 2.02] was statistically not distinguishable from zero. Moreover, changes in the protein content of the cultures as a result of experimental manipulation of the cultures also showed a significant correlation with changes in the DNA content of the cultures ($r = 0.849$; $P < 0.001$). Again, the intercept of the regression line was not different from zero.

Combined effects of Buserelin and oestradiol

Subsequently, the possibility that the LHRH analogue interferes with the action of oestradiol on the cells was investigated. The results presented in Fig. 2 show that although the stimulatory effect of oestradiol on the cells is rather small, addition of Buserelin combined with oestradiol results in a significantly lower protein and DNA content than addition of oestradiol alone. The inhibitory effect of Buserelin on the protein content of MCF-7 cultures in the presence of oestradiol was dependent on the dose of the peptide (Fig. 3). Moreover, LHRH itself also showed a slight anti-proliferative effect (Fig. 3).

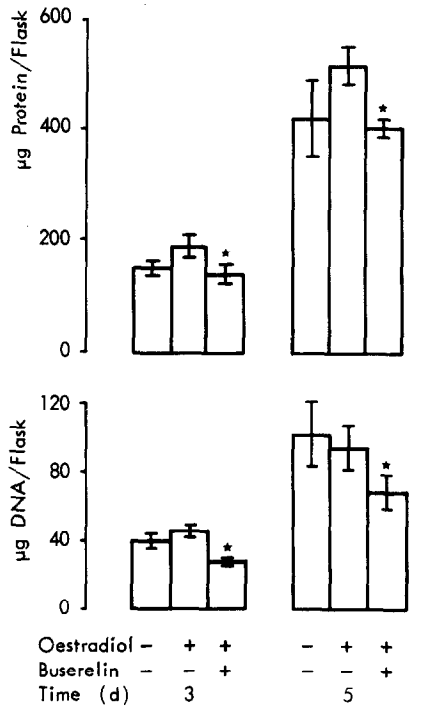

Fig. 2. Protein and DNA content of MCF-7 cultures grown in fully supplemented medium at different times during daily administration of 1 nM *oestradiol alone or in combination with* 80 nM *Buserelin. Results are means ± S.D.;* n = 4–6; *P < 0.02 *vs cultures kept in the presence of oestradiol only.*

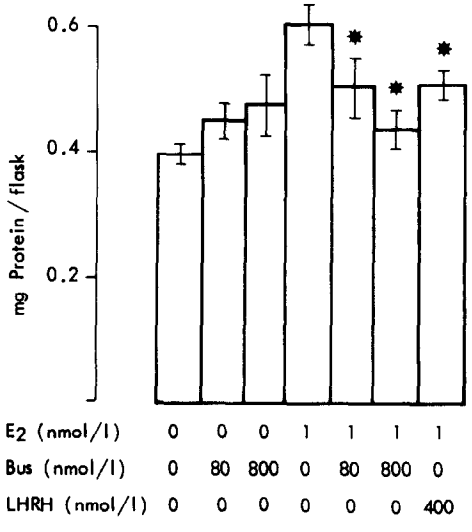

Fig. 3. Protein content of MCF-7 cultures grown in fully supplemented medium after five daily administrations of oestradiol (E_2) alone or combined with 80 *or* 800 nM *Buserelin (Bus) or* 400 nM *synthetic LHRH. Results are given as means ± S.D.;* n = 8–9; *P < 0.01 *vs cultures kept in the presence of oestradiol alone.*

Effect of an LHRH antagonist and steroid-depleted medium

The data in Fig. 4 demonstrate that the effect of Buserelin on oestrogen-stimulated MCF-7 cells can be counteracted by an equimolar amount of the LHRH antagonist ORG 30093D. This com-

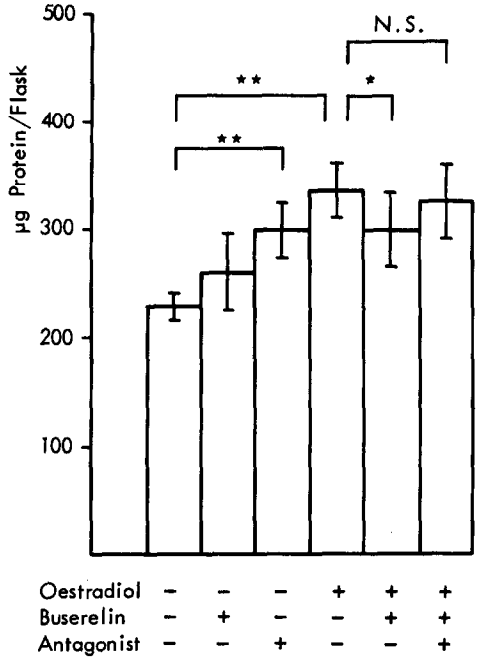

Fig. 4. Protein content of MCF-7 cells kept for 5 days *in fully supplemented medium containing oestradiol* (1 nM), *Buserelin* (800 nM) *or the LHRH antagonist ORG* 30093*D* (800 nM), *either alone or in combination. Culture medium was changed daily. Results are given as means ± S.D.;* n = 10; *P < 0.05; **P < 0.01.

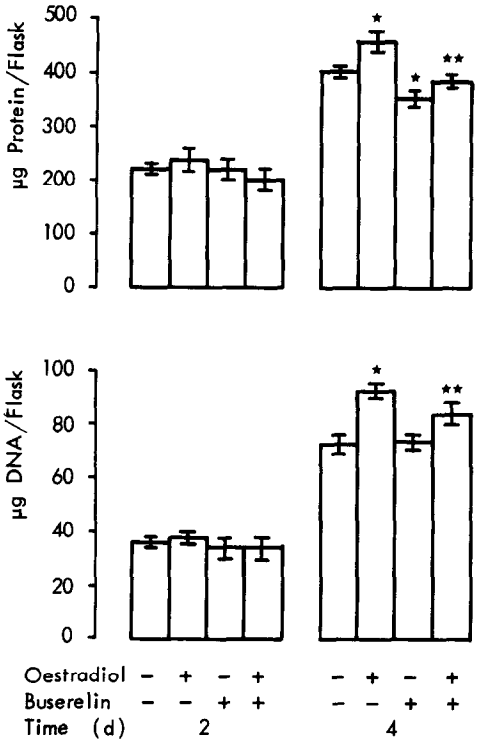

Fig. 5. Protein and DNA content of MCF-7 cultures kept in medium containing 10% *DCCFCS for different periods of time. Cells were trypsinized and transferred to T-25 flasks in fully supplemented medium. After* 1 day *(at day 0), this medium was replaced by the steroid-depleted medium containing* 1 nM *oestradiol and* 800 nM *Buserelin either alone or in combination. Results are given as means ± S.D.;* n = 7; *P < 0.01 *vs control cultures.* **P < 0.01 *vs cultures kept in the presence of oestradiol alone.*

pound also appeared to be able to stimulate the growth of the MCF-7 cells.

Effects of oestradiol and Buserelin may have been reduced by interference from compounds present in FCS. The results in Fig. 5 show that in medium prepared with steroid-depleted FCS Buserelin also inhibits the oestradiol-induced increases in cellular protein and DNA.

LHRH binding studies

The binding of radio-iodinated LHRH to MCF-7 cells and rat pituitary membranes is shown in Fig. 6. Pituitary membranes readily bound the tracer, which could be displaced from its binding sites by an excess of radio-inert LHRH or Buserelin. Scatchard plot analysis of the binding of radioactive LHRH to rat pituitary membranes revealed a binding capacity of 40 fmol/mg membrane protein and a dissociation constant of 0.06 nM. By contrast, the tracer bound to the MCF-7 cells to a much smaller extent, and the binding observed could not be displaced by an excess of radioinert ligand. No binding was observed to membranes prepared from solid human mammary and ovarian tumours.

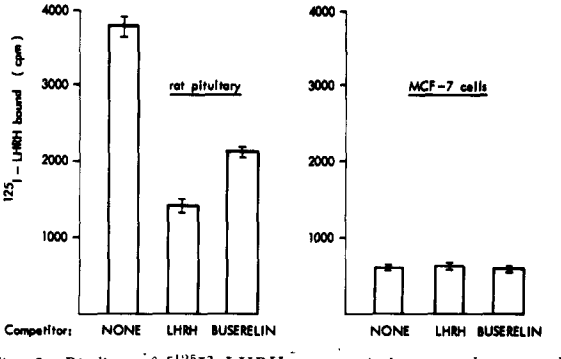

Fig. 6. Binding of [^{125}I]*-LHRH to rat pituitary membranes and MCF-7 cells and displacement by a* 1000*-fold excess of radioinert LHRH or an* 8000*-fold excess of Buserelin. Results are given as means ± S.D.;* n = 3 *for the pituitary membranes;* n = 5 *for the MCF-7 cells.*

DISCUSSION

The results described in the present paper are the first to demonstrate that an LHRH agonist can directly interfere with the proliferation of human breast cancer cells in culture. It appears from our data that Buserelin can antagonize the stimulatory action of oestrogens on these cells. LHRH itself was also capable of inhibiting the oestradiol-induced proliferation of the cells, whereas the LHRH antagonist ORG 30093D counteracted the effect of Buserelin. The limited magnitude of the effects of Buserelin on the cells, however, supports the current consensus that the main anti-tumour effect of LHRH analogues is exerted via a suppression of circulating levels of oestrogens.

The reason why only relatively small effects of

Buserelin were observed may very well reside in the fact that oestradiol also had limited effects. Yet the magnitude of the stimulatory effect of oestradiol on our MCF–7 cells is not different from that in other reports [30–32]. Other investigators have shown that the effect of oestradiol on these cells may depend on the source of serum [33]. It is conceivable that the anti-proliferative action of Buserelin is more pronounced in cells which show a higher increase in growth rate in response to oestradiol. Therefore the use of other sera is currently under investigation.

One attempt to increase the sensitivity of the cells to oestradiol was already undertaken in the present series of experiments. The presence of FCS in the culture medium may result in an oestrogen concentration which already causes a maximal or near-maximal proliferation rate of the cells. Alternatively, other mitogens present in FCS may stimulate the proliferation of the cells to such an extent that they cannot respond to addition of oestradiol with an increase in the rate of proliferation. Therefore experiments were also done with medium in which the FCS was replaced with DCCFCS. The rates of both the basal and the oestradiol-induced proliferation in medium prepared with DCCFCS (Fig. 5) decreased only marginally as compared with medium prepared with untreated FCS (Fig. 2), which led us to conclude that other factor(s) present in FCS and resistant to charcoal treatment must be responsible for maintaining the proliferation rate of the cells on a relatively high level. It could be argued that the action of Buserelin on the MCF–7 cells is directed against the stimulatory action of these other mitogens, rather than oestradiol. In our opinion, however, this possibility is not very likely since Buserelin had no effect in the absence of oestradiol.

Our data are in agreement with those of Corbin [19], who found that daily administration of the LHRH agonist Wy 40,972 temporarily retarded the growth of mouse mammary tumours cells in culture. In contrast to our results, however, Corbin observed an effect already 1 day after the first administration, and after 6 days cell numbers in cultures treated with the analogue were equal to those in control cultures. In our study the first effects could be shown only after 3–4 days. In view of the relatively high growth rate of the cells, effects could not be investigated for more than 6 days. Therefore we can offer no data on the duration of the suppression of cell growth. From the data in Figs 2 and 5, however, it appears that the effect of Buserelin on the MCF–7 cells is also transient. In the experiment reported in Fig. 2, for example, the protein content of control cultures and cultures kept in the presence of oestradiol or oestradiol plus Buserelin all increased by a factor of 2.7 between days 3 and 5. The inhibitory action of Buserelin thus appears to be manifested only after initial exposure of the cells to the peptide. The most obvious explanation for this observation is inactivation of the peptide by degradative enzymes. To circumvent this possibility, the culture medium was replaced daily in the present series of experiments. To further evaluate this possibility, an experiment was done in which the culture medium was not changed. Medium which is conditioned by MCF–7 cells for more than 5 days can still stimulate the secretion of LH and FSH by rat pituitary cells in culture. In this respect there was no difference between fresh and conditioned medium [F.H. de Jong, personal communication]. The presence of biological LHRH activity after prolonged exposure of the medium to MCF–7 cells virtually rules out the possibility that the transiency of the effect of Buserelin on the cells is caused by exhaustive degradation of the peptide.

Other explanations for this phenomenon include transient effects on cell attachment, changes in the uptake of the peptide by the cells, the existence of several populations of cells, of which only a minority is sensitive to Buserelin, or the secretion of an LHRH-like peptide by the cells. The secretion of an endogenous LHRH-like regulatory peptide may, of course, obscure effects of exogenously added peptides. The presence of high concentrations of LHRH-like immunoreactive material has been documented in human milk [34, 35] and in ductal mammary carcinoma [36]. It remains to be investigated which, if any, of these possibilities accounts for the present observations.

In the present study the growth-inhibiting effect of Buserelin was tested at only one, relatively high concentration of oestradiol, i.e. 1 nM. This concentration was chosen because it is known to induce maximal stimulation of MCF–7 cells. It remains to be investigated whether the growth-inhibiting effect of Buserelin is dependent on the concentration of oestradiol. To answer this question it may be essential to use a system in which the response of the cells to oestradiol is much more pronounced.

Our observation that Buserelin and LHRH itself can act directly on MCF–7 cells is highly suggestive for the presence of specific receptors for LHRH-like peptides in these cells. A similar situation appears to prevail for the Dunning R 3327H rat prostatic carcinoma. This transplantable tumour regresses following administration of LHRH analogues [25]. Recently, this tumour was also found to contain receptors for LHRH-like peptides [37]. We have used commercially available iodinated LHRH in the search for the presence of such receptors in MCF–7 cells. No saturable binding of labelled LHRH to the cells was observed. By contrast, rat pituitary membranes showed specific binding of the peptide. This virtually rules out the possibility that the LHRH lost

its biological activity upon iodination, but indicates that the putative receptors for LHRH-like peptides in MCF-7 cells may have a very low affinity for LHRH itself. Alternatively, the observed effects of LHRH on the MCF-7 cells (Fig. 3) need not be mediated through receptors, or putative receptors may be occupied with endogenous LHRH-like material [34–36]. After submission of this paper, data were published by Miller *et al.* [38], which support the suggestion that the putative receptors for LHRH-like material have a low affinity for native LHRH. These authors found that iodinated LHRH-agonist was able to bind to MCF-7 cells, but that LHRH itself showed only a very limited cross-reactivity with this binding.

In summary, the data reported in the present paper support the hypothesis that LHRH agonists, apart from their main antitumour activities which are mediated by the pituitary and the gonads, may have an additional antitumour effect excerted directly on the tumour cells.

Acknowledgements—We thank Hoechst AG, Frankfurt, F.R.G., and the Medical Department of Hoechst Holland BV for supplies of the LHRH agonist Buserelin. We acknowledge the cooperation with Dr A.V. Schally, and Organon International, Oss, The Netherlands, who made the LHRH antagonist ORG 30093D available. We are grateful to Dr F.H. de Jong and Dr F.F.G. Rommerts, Department of Biochemistry II, Erasmus University Rotterdam, for testing the LHRH-bioactivity of culture media and critically reviewing the manuscript respectively.

REFERENCES

1. Klijn JGM, de Jong FH. Treatment with a luteinising hormone-releasing hormone analogue (Buserelin) in premenopausal patients with metastatic breast cancer. *Lancet* 1982, **i**, 1213–1216.
2. Klijn JGM, de Jong FH, Blankenstein MA *et al.* Anti-tumor and endocrine effects of chronic LHRH agonist (Buserelin) treatment with or without tamoxifen in premenopausal metastatic breast cancer. *Breast Cancer Res Treat* 1984, **4**, 209–220.
3. Borgmann V, Hardt W, Schmidt-Gollwitzer M, Adenauer H, Nagel R. Sustained suppression of testosterone production by the luteinising-hormone releasing-hormone agonist Buserelin in patients with advanced prostate carcinoma. *Lancet* 1982, **i**, 1097–1099.
4. Klijn JGM, de Jong FH, Lamberts SWJ, Blankenstein MA. LHRH-Agonist treatment in metastatic prostate carcinoma. *Eur J Cancer Clin Oncol* 1984, **20**, 483–493.
5. Schally AV, Redding TW, Comaru-Schally AM. Potential use of analogs of luteinizing hormone-releasing hormones in the treatment of hormone-sensitive neoplasms. *Cancer Treat Rep* 1984, **68**, 281–289.
6. Schally AV, Arimura A, Coy DH. Recent approaches to fertility control based on derivatives of LH–RH. *Vit Horm* 1980, **38**, 257–323.
7. Gonzalez-Barcena D, Kastin AJ, Coy DH, Nikolics K, Schally AV. Suppression of gonadotrophin release in man by an inhibitory analogue of L.H.-releasing hormone. *Lancet* 1977, **ii**, 997–998.
8. Sandow J. Clinical applications of LHRH and its analogues. *Clin Endocrinol* 1983, **18**, 571–592.
9. Clayton RN, Harwood JP, Catt KJ. Gonadotropin-releasing hormone analogue binds to luteal cells and inhibits progesterone production. *Nature* 1979, **282**, 90–92.
10. Hsueh AJW, Erickson GF. Extrapituitary action of gonadotropin-releasing hormone: direct inhibition of ovarian steroidogenesis. *Science* 1979, **204**, 854–855.
11. Sharpe RM, Fraser HM, Cooper I, Rommerts FFG. Sertoli-leydig cell communication via an LHRH-like factor. *Nature* 1981, **290**, 785–787.
12. Labrie F, Seguin C, Lefebvre FA *et al.* Gonadal LHRH receptors and direct gonadal effects of LHRH agonists. *Front Horm Res* 1982, **10**, 33–42.
13. Pieper DR, Richards JS, Marshall JC. Ovarian gonadotropin-releasing hormone (GnRH) receptors: characterization, distribution and induction by GnRH. *Endocrinology* 1981, **108**, 1148–1155.
14. Ranta T, Knecht M, Kody M, Catt KJ. GnRH receptors in cultured rat granulosa cells: mediation of the inhibitory and stimulatory actions of GnRH. *Mol Cell Endocrinol* 1982, **27**, 233–240.
15. Tureck RW, Mastroianni L, Blasco L, Strauss JF. Inhibition of human granulosa cell progesterone secretion by a gonadotropin-releasing hormone agonist. *J Clin Endocrinol Metab* 1982, **54**, 1078–1080.
16. Casper RF, Erickson GF, Rebar RW, Yen SSC. The effect of luteinizing hormone-releasing factor and its antagonist on cultured human granulosa cells. *Fertil Steril* 1982, **37**, 406–409.
17. Clayton RN, Huhtaniemi IT. Absence of gonadotropin-releasing hormone receptors in human gonadal tissue. *Nature* 1982, **299**, 56–59.
18. Popkin R, Bramley TA, Currie A, Shaw RW, Baird DT, Fraser HM. Specific binding of luteinizing hormone releasing hormone to human luteal tissue. *Biochem Biophys Res Commun* 1983, **114**, 750–756.
19. Corbin A. From contraception to cancer: a review of the therapeutic applications of LHRH analogues as antitumour agents. *Yale J Biol Med* 1982, **55**, 27–47.

20. Lecomte P, Wang N-G, Sundaram K, Rivier J, Vale W, Bardin CW. The antiandrogenic action of gonadotropin-releasing hormone and its agonists on the mouse kidney. *Endocrinology* 1982, **110**, 1–6.
21. Sundaram K, Cao Y-Q, Wang N-G, Bardin CW, Rivier J, Vale W. Inhibition of the actions of sex steroids by gonadotropin-releasing hormone (GnRH) agonists: a new biological effect. *Life Sci* 1981, **28**, 83–88.
22. Pedroza E, Vilchez-Martinez JA, Coy DH, Arimura A, Schally AV. Reduction of LHRH pituitary and estradiol uterine binding sites by a superactive analog of luteinizing hormone-releasing hormone. *Biochem Biophys Res Commun* 1980, **95**, 1056–1062.
23. Rao IM, Reddy PRK. Direct inhibitory effect of gonadotropin releasing hormone in the uterus of rat. *Life Sci* 1984, **34**, 2257–2263.
24. Furr BJA, Nicholson RI. Use of analogues of luteinizing hormone-releasing hormone for the treatment of cancer. *J Reprod Fertil* 1982, **64**, 529–539.
25. Redding TW, Coy DH, Schally AV. Prostate carcinoma tumor size in rats decreases after administration of antagonists of luteinizing hormone-releasing hormone. *Proc Natl Acad Sci USA* 1982, **79**, 1273–1276.
26. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976, **72**, 248–254.
27. Setaro F, Morley CGD. A modified fluorimetric method for the determination of microgram quantities of DNA from cell or tissue cultures. *Anal Biochem* 1976, **71**, 313–317.
28. Alabaster O, Vonderhaar BK, Shafie SM. Metabolic modification by insulin enhances methotrexate cytotoxicity in MCF–7 human breast cancer cells. *Eur J Cancer Clin Oncol* 1981, **17**, 1223–1228.
29. Loumaye E, Naor Z, Catt KJ. Binding affinity and biological activity of gonadotropin-releasing hormone agonists in isolated pituitary cells. *Endocrinology* 1982, **111**, 730–736.
30. Butler WB, Kelsey WH, Goran N. Effects of serum and insulin on the sensitivity of the human breast cancer cell line MCF–7 to estrogen and antiestrogens. *Cancer Res* 1981, **41**, 82–88.
31. Lippman M, Bolan G. Oestrogen-responsive human breast cancer in long-term tissue culture. *Nature* 1975, **256**, 592–593.
32. Sutherland RL, Green, MD, Hall RE, Reddel RR, Taylor IW. Tamoxifen induces accumulation of MCF–7 human mammary carcinoma cells in the G_0/G_1 phase of the cell cycle. *Eur J Cancer Clin Oncol* 1983, **19**, 615–621.
33. Page MJ, Field JK, Everett NP, Green CD. Serum regulation of the estrogen responsiveness of the human breast cancer cell line MCF–7. *Cancer Res* 1983, **43**, 1244–1250.
34. Amarant T, Fridkin M, Koch Y. Luteinizing hormone-releasing hormone and thyrotropin releasing hormone in human and bovine milk. *Eur J Biochem* 1982, **127**, 647–650.
35. Sarda AK, Nair RMG. Elevated levels of LRH in human milk. *J Clin Endocrinol Metab* 1981, **52**, 826–828.
36. Seppällä M, Wahlström T. Identification of luteinizing hormone releasing factor and alpha subunit of glycoprotein hormones in ductal carcinoma of the mammary gland. *Int J Cancer* 1980, **26**, 267–268.
37. Hierowski MT, Altamirano P, Redding TW, Schally AV. The presence of LHRH-like receptors in Dunning R3327H prostate tumors. *FEBS Lett* 1983, **154**, 92–96.
38. Miller WR, Scott WN, Morris R, Fraser HM, Sharpe RM. Growth of human breast cancer cells inhibited by a luteinizing hormone-releasing hormone agonist. *Nature* 1985, **313**, 231–233.