Inhibition of growth of human mammary tumor cells by potent antagonists of luteinizing hormone-releasing hormone (cell proliferation/[^3H]thyymidine incorporation/MDA-MB-231 breast cancer line)

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ABSTRACT Various studies support the view that analogs of luteinizing hormone-releasing hormone (LH-RH) exert some direct effects on mammary tumor cells. Recently, new LH-RH antagonists [Ac-d-Nal(2),d-Phe(pCl)3,D-Trp4,D-Hci6,D-Ala10]-LH-RH (SB-29) and [Ac-d-Nal(2),D-Phe(pCl)2,D-Trp3,D-Cit4,D-Ala10]-LH-RH (SB-30), which are devoid of edematogenic effects, were synthesized. In this study, we examined whether these LH-RH antagonists inhibit the proliferation of MDA-MB-231 human mammary tumor cells in culture. [^3H]Thymidine incorporation into DNA and cell number were measured. The antagonists induced up to 40% inhibition of [^3H]thymidine incorporation in MDA-MB-231 cells. This inhibition was dose-dependent in the 0.3–30 μM range and could be demonstrated after 2 days of incubation in the presence of the peptides. An older antagonist, [Ac-d-Phe(pCl)1,2,D-Trp4,D-Arg6,D-Ala10]-LH-RH (ORG 30276), had a lesser effect, and the agonist des-Gly10[D-Ser(Bu)8]-LH-RH ethylamide (buserelin) had no effect. The antagonists SB-29 and SB-30 also inhibited the rate of cell growth, as measured by cell number, while the LH-RH agonist buserelin had no significant effect. These results support the concept that these new LH-RH antagonists can directly inhibit the growth of human mammary tumors and thus might be suitable for the treatment of breast cancer.

Chronic administration of potent agonists of luteinizing hormone-releasing hormone (LH-RH) results in the inhibition of pituitary and gonadal function (1,2) and creates a state of sex-steroid deficiency. Consequently, agonists of LH-RH can be used for the treatment of some hormone-dependent tumors, such as prostatic and breast cancer (1,2). In view of many expected medical applications of LH-RH derivatives, more than 2000 analogs have been synthesized since the isolation and structural elucidation of LH-RH (3). Many agonistic analogs are more potent than the parent hormone have been made (1,2,4). Several of these agonists are being used clinically (1,2,5). Potent inhibitory analogs of LH-RH, which block ovulation in laboratory animals, have also been synthesized (4). Several antagonists of LH-RH have been tested in men and women and shown to be active enough for practical use (4). LH-RH antagonists may offer certain advantages in the treatment of cancer as compared with the superagonists. While a repeated administration of LH-RH agonists is required to inhibit the pituitary and gonadal function and reduce the levels of sex steroids, the same effect may be obtained by a single administration of LH-RH antagonists (4). The inhibition of gonadotropin release by LH-RH antagonists starts immediately after its administration, while the agonists cause a transient stimulation of pituitary and gonadal function, which may result in a temporary clinical "flare-up" of the disease (5).

LH-RH antagonists are frequently characterized by the nature of the residue at position 6. Some potent LH-RH antagonists contained D-arginine (hydrophilic basic substitution) at this position. However, this class of analogs produced transient edema of the face and extremities when administered subcutaneously to rats (6,7). In preliminary human tolerance studies, side effects were also observed in some cases after administration of antagonists with basic D-amino acids at position 6. These reactions could be due to histamine liberation. These side effects delayed clinical use of LH-RH antagonists in humans.

To obtain LH-RH antagonists free of edematogenic effects, new analogs with D-ureidoalkyl amino acids, such as D-citrulline (D-Cit) and D-homocitrulline (D-Hci), at position 6 were synthesized and tested in several in vitro and in vivo systems (4). Synthesis of [D-Cit][LH-RH and [D-Hci][LH-RH analogs of LH-RH produced very potent antagonists as judged by the inhibition of LH release in vivo, blockade of ovulation in cycling rats, and suppression of LH levels in ovariectomized rats. Characteristically, these peptides did not have any edematogenic effects, even at high doses (4).

Conflicting data exist on the possible direct effect of various LH-RH analogs on the growth of mammary tumor cells in culture. Miller et al. (8) proposed that LH-RH and some of its agonists may inhibit the growth of MCF-7 mammary tumor cells in culture. However, Eidne et al. have not been able to confirm these results (9): they reported that LH-RH agonists had no significant effect on cell growth, but some antagonists inhibited [^3H]thymidine incorporation in human breast cancer cells (9).

In the present study, we evaluated the effects of two antagonists free of edematogenic effects: [Ac-d-Nal(2),d-Phe(pCl)3,d-Trp4,d-Hci6,d-Ala10]-LH-RH (SB-29) and [Ac-d-Nal(2),d-Phe(pCl)2,d-Trp3,d-Cit6,d-Ala10]-LH-RH (SB-30) on the growth of human mammary tumor cells in culture. These effects were compared with those of an older antagonist, [Ac-d-Phe(pCl)1,2,d-Trp4,d-Arg6,d-Ala10]-LH-RH (ORG 30276), and a well-known superagonist, des-Gly10[D-Ser(Bu)8]-LH-RH ethylamide (buserelin).

MATERIALS AND METHODS

Peptides. Buserelin was a gift from J. Sandow (Hoechst), ORG 30276 was obtained from L. Tax (Organon), the antagonists SB-29 and SB-30 were synthesized and purified in the Veterans Administration/Tulane University Laboratory as reported (4).

Abbreviations: LH, luteinizing hormone; LH-RH, luteinizing hormone-releasing hormone; SB-29, [Ac-d-Nal(2),d-Phe(pCl)3,d-Trp4,d-Hci6,d-Ala10]-LH-RH; SB-30, [Ac-d-Nal(2),d-Phe(pCl)2,d-Trp3,d-Cit6,d-Ala10]-LH-RH; ORG 30276, [Ac-d-Phe(pCl)1,2,d-Trp4,d-Arg6,d-Ala10]-LH-RH; Cit, citrulline; Hci, homocitrulline; Nal(2), 3(2-naphthyl)-alanine; DMBA, 7,12-dimethylbenz(a)anthracene; Phe(pCl), 4-chlorophenylalanine.
Cells. MDA-MB-231 human mammary cancer cells were obtained from H. Rochefort (Institut National de la Santé et de la Recherche Médicale, Montpellier, France). The cells were grown in 75-cm² flasks in Dulbecco's modified Eagle's medium (Biological Industries, Beth Haemek, Israel) containing penicillin (100 units/ml), streptomycin (0.1 mg/ml), nystatin (12.5 units/ml), and 10% (vol/vol) fetal calf serum.

\(^{3}H\)Thymidine Incorporation. Cells were seeded into 96-multiwell plates in 0.1 ml of medium. After 1 day, the medium was replaced with medium containing various concentrations of LH-RH analogs and changed daily. After 2 or 3 days of incubation, fresh medium was used containing \(^{3}H\)thymidine (40 \(\mu\)Ci/ml; 1 \(\mu\)Ci = 37 kBq), in addition to the peptides. The incorporation was stopped by addition of unlabeled thymidine (0.5 \(\mu\)mol). The cells were washed twice with phosphate-buffered saline and three times with 10% trichloroacetic acid to remove soluble thymidine and then were dissolved in 0.1 M NaOH. The basic solution was neutralized with 5% vol/vol acetic acid and assayed in Insta-Gel (Packard, Downers Grove, IL). Cbr

Cell Proliferation Experiments. Cells were seeded into 6-multiwell plates (4 \(\times\) 10\(^4\) cells per well) and grown for 1 day. The medium was then replaced with fresh medium containing the LH-RH analogs and changed daily. At the times indicated, the cells were removed with trypsin and counted with a hemacytometer.

**RESULTS**

Effect of Incubation Time and Cell Number on \(^{3}H\)Thymidine Incorporation in MDA-MB-231 Human Mammary Cancer Cells. \(^{3}H\)Thymidine incorporation into cellular trichloroacetic acid-precipitable material is a widely accepted method for evaluating the rate of DNA synthesis. To establish the optimal experimental condition in MDA-MB-231 cells, the effects of cell number and the duration of the incubation with the radioactive compound were evaluated. The results (Fig. 1) indicate that \(^{3}H\)thymidine incorporation in MDA-MB-231 mammary cancer cells was linear up to 10,000 cells per well. The subsequent experiments were conducted within these limits.

Inhibition of \(^{3}H\)Thymidine Incorporation in MDA-MB-231 Cells by LH-RH Analogos. The effects of four LH-RH analogs on \(^{3}H\)thymidine incorporation in mammary tumor cells were studied. The analogs tested included two new LH-RH antagonists SB-29 and SB-30, the potent antagonist, ORG 30276, and the superagonist buserelin. The cells were exposed for 1 or 2 days to different concentrations of the peptides which ranged between 3 nM and 30 \(\mu\)M. After 1 day of exposure, only a small inhibition was seen with the three antagonists at high concentrations (Fig. 2). However, on day 2, a significant inhibition by the three antagonists was obtained. The new antagonists SB-29 and SB-30 were significantly more potent than ORG 30276 and produced a similar inhibition of \(^{3}H\)thymidine incorporation at doses 1/10th to 1/100th those required for ORG 30276. Buserelin had no significant effect. When the effect of LH-RH analogs was investigated in estrogen-dependent MCF-7 mammary cancer cell line, preliminary results indicated that SB-29 and SB-30 inhibited \(^{3}H\)thymidine incorporation (not shown).

The results of \(^{3}H\)thymidine incorporation into trichloroacetic acid-precipitable DNA material may not fully represent cell growth in all cell lines. For this reason we decided to check the effects of the new antagonists on cell proliferation by measuring cell number directly.

Inhibition of MDA-MB-231 Proliferation by SB-29 and SB-30. Cell number was monitored over a period of several days when the cells were incubated in the presence of SB-29 and SB-30. The doubling time of the control cells was about 1 day. This high growth rate was suppressed by both antagonists (Fig. 3). The growth inhibition was detectable from day 2 of exposure to antagonists and reached a maximal level of 84% on day 5 of treatment with 30 \(\mu\)M SB-29. Although this is a high concentration, there was also a considerable inhibition at 10 \(\mu\)M (60%) and 10 \(\mu\)M (53%). The inhibition of cell growth by SB-30 appeared to be smaller than that caused by SB-29. Since this difference was evident only on or after the third day of incubation, it is possible that the two compounds possess different stabilities in the incubation medium. The LH-RH agonist buserelin did not affect cell growth significantly (Fig. 4), although a slight inhibition may occur after 6 days of incubation.

**DISCUSSION**

Previous in vivo and in vitro results support the view that LH-RH analogs have a direct effect on mammary tumors.
Specific receptors for LH-RH analogs in mammary tumors have been detected by Eidne et al. (10) and Fekete et al. (11). The presence of LH-RH immunoreactivity and mRNA for this peptide (12) in human mammary cancer cells suggests that LH-RH may play a role in the growth of mammary tumors. Thus, the idea that LH-RH antagonists may interfere with the postulated effect of LH-RH on growth of mammary cancer cells is appealing. The results presented here show that the new LH-RH antagonists, which are highly effective in displacing bound [d-Trp<sup>6</sup>]LH-RH from mammary tumor membranes (13), suppress DNA synthesis by mammary cancer cells as measured by [<sup>3</sup>H]thymidine incorporation in MDA-MB-231 cells. These antagonists also cause a marked inhibition of cell growth as indicated by cell number. If these antagonists inhibit cancer cell growth by nullifying the action of endogenous LH-RH or a related peptide, then in analogy with the inhibitory effects of agonists on the pituitary, this action could also be suppressed by an LH-RH agonist. Such an effect of LH-RH agonists was reported by Miller et al. in MCF-7 cells (8) but was not found in another study with the same cell line or with MDA-MB-231 cells (9). Similarly, in our study the LH-RH agonist buserelin did not affect [<sup>3</sup>H]thymidine incorporation or cell growth in MDA-MB-231 cells. The lack of effect could be explained by a difference in the desensitization of the LH-RH receptors in these cells. An alternative explanation might be that the LH-RH antagonists could inhibit cell growth by another mechanism or by interacting with additional receptors.

A smaller effect of ORG 30276 on [<sup>3</sup>H]thymidine incorporation as compared with that of the new antagonists SB-30 and SB-29 suggests that this new class of antagonists (4) might be better for the treatment of breast cancer than the analogs previously available. In addition, these new antagonists were shown to be devoid of the edematogenic effects characteristic of the [d-Arg<sup>6</sup>]LH-RH analogs (4). An inhibition of [<sup>3</sup>H]thymidine incorporation in human breast cancer lines by various LH-RH antagonists were also shown by Eidne et al. (9). These antagonists contained d-arginine at position 6 and caused a rapid inhibition of [<sup>3</sup>H]thymidine incorporation. However, when one of these antagonists was tested in a cell-growth experiment, it proved to be much less effective and less potent in inhibiting cell proliferation (9) than the antagonists studied by us. The discrepancy between thymidine incorporation and cell growth in experiments by Eidne et al. (9) is not clear.

For our studies, we used the hormone-independent human cell line MDA-MB-231. This line is characterized as estrogen unresponsive (14); thus, it may be a good model for testing the antagonists of LH-RH for their direct inhibitory effect, which must be clearly differentiated from the antitumor effects produced by the creation of the state of estrogen deficiency. The MDA-MB-231 line may be representative of many human mammary tumors. These ‘‘estrogen-independent’’ breast cancers are less responsive to various hormonal manipulations, are frequently more aggressive in nature (15), and thus present a challenge for the development of drugs that might affect their growth. A direct effect of LH-RH analogs on estrogen-responsive 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumor in the rat has been demonstrated by us (16). The DMBA-induced mammary carcinoma serves as a model for the human hormone-responsive cancers, as its growth rate can be influenced by endocrine manipulations. Estrogens (17) and prolactin (18) stimulate the growth of DMBA tumors, while ovariectomy (17), antiestrogens (19), and drugs that decrease plasma prolactin levels cause growth arrest or regression of these tumors (20). Moreover, we have shown that hormonal transmembrane transducing mechanisms, such as tyrosine kinase activity (20) and the phosphatidylinositol cycle (21), may be involved in the growth control of DMBA tumors. A direct interaction of LH-RH analogs with DMBA tumor was shown by the enhancement of phospholipase C activity in the tumor membranes by LH-RH agonists and the inhibition of this activity by LH-RH antagonists (16). Thus, there is a high probability that human sex-steroid-responsive mammary tumors would respond to treatment with LH-RH antagonists not only by virtue of estrogen deprivation but also through direct effects. Preliminary results with [<sup>3</sup>H]thymidine incorporation in the estrogen-responsive MCF-7 cells support the notion of direct effects. The inhibition of estrogen-dependent mammary tumors in mice and rats by administration of LH-RH antagonists, including ORG 30276, has been documented (22).

Our study shows that the new LH-RH antagonists with d-citrulline and d-homocitrulline at position 6 are better inhibitors of MDA-MB-231 mammary tumor cell proliferation than the 6-d-arginine-substituted antagonist ORG 30276 or the superagonist buserelin. In view of favorable clinical results with LH-RH agonists in the treatment of prostate cancer, mammary carcinomas, and ovarian cancer (1, 2), our results support the view that the new LH-RH antagonists also might be potentially useful for the therapy of these and other tumors.

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