Recovery of pituitary function after treatment with a targeted cytotoxic analog of luteinizing hormone-releasing hormone

(targeted chemotherapy/pituitary hormones/growth hormone/thyrotropin/selective damage)

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ABSTRACT Recently, we developed a targeted cytotoxic analog AN-207 of luteinizing hormone-releasing hormone (LH-RH), consisting of an intensely potent derivative of doxorubicin, 2-pyrrolindodoxorubicin (AN-201) conjugated to carrier agonist [d-Lys6]LH-RH. In this study, we investigated the effects of cytotoxic analog AN-207, designed for targeted chemotherapy and radical AN-201 on pituitary function in rats. A selective damage to the pituitary gonadotroph cells was found at 1 week after a single i.v. injection of 150 nmol/kg AN-207, as evidenced by a 63% decrease in the LH-RH-stimulated release of LH in vitro. The release of growth hormone (GH) and thyrotropin (TSH), stimulated by GH-releasing hormone (GH-RH) and TSH-releasing hormone (TRH), respectively, was reduced by only 11–12%. In contrast, even a smaller dose of 75 nmol/kg of AN-201 nonselectively damaged pituitary function, reducing the stimulated release of LH, GH, and TSH by 57%, 74%, and 67%, respectively. Two weeks after administration, the LH-RH-stimulated LH release in vivo entirely normalized in the AN-207-treated rats, and only a 15% decrease in the LH response was found in the group given AN-201. GH and TSH responses to receptor-mediated stimuli with GH-RH and TRH were normal at 2 weeks in both treated groups. Neither cytotoxic compound caused changes in the concentration of pituitary LH, GH, or TSH, as determined by RIA at 1 week and 7 weeks after treatment. This study demonstrates that the cytotoxic LH-RH analog AN-207 exerts highly selective effects on the gonadotroph cells containing LH-RH receptors and is less toxic for other cells. Conversely, its cytotoxic radical AN-201 nonselectively damages the pituitary cells. The damaging effect of both cytotoxic compounds on pituitary functions is reversible. In view of its high selectivity and reduced toxicity, AN-207 could be a potential therapeutic agent for the treatment of tumors that possess receptors for LH-RH such as prostatic, mammary, ovarian, and endometrial cancers.

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RH), and the carrier [D-Lys₆]LH-RH were synthesized and characterized in our laboratory as reported (17, 18). LH-RH and GH-RH(1-29)NH₂ were also synthesized in our laboratory, while TRH was obtained from Takeda (Osaka).

**Experimental Procedure.** Female adult Sprague–Dawley rats (200–250 g) were used for all experiments. The rats were maintained under controlled conditions of lighting (12-h light, 12-h dark schedule) and temperature (24 ± 2°C) with free access to standard rat chow pellets and tap water.

**Treatment and in vivo tests.** These experiments were designed to investigate the function of the pituitary gland at various time periods after the treatment with cytotoxic LH-RH analog AN-207 or its cytotoxic radical AN-201. In experiment 1, the rats were divided into four groups of 10 animals. Group 1 received a single i.v. injection of 150 nmol/kg AN-207, group 2 was injected with 75 nmol/kg AN-201, and control rats in groups 3 and 4 were treated with 150 nmol/kg carrier [D-Lys₆]LH-RH or saline. All injections were performed under methoxyflurane (Metofane; Pittman-Moore, Mundelein, IL) anesthesia. The doses of AN-207 and AN-201 used were previously found to be the maximum tolerated doses inducing growth inhibition of Dunning R-3327-H, androgen dependent prostate cancer in rats (19). All compounds were dissolved in 0.9% sodium chloride and injected into the jugular vein. Body weights (BW) of the animals were recorded once a week, and vaginal smears were examined every day. Two, 4, and 6 weeks after the treatment, specific receptor-mediated responsiveness of pituitary to various releasing hormones was tested by injecting the mixture of 1 µg LH-RH, 1 µg GH-RH, and 1 µg TRH into the jugular vein of six rats in each group. Blood samples were obtained from the jugular vein under Metofane anesthesia before the injection (0 min) and 5, 60, and 180 min later. The volume of blood taken was replaced by saline. The blood was centrifuged, and sera were stored at −20°C until assayed for LH, GH, and TSH by RIA. Seven weeks after the treatment with the cytotoxic compounds, the rats were decapitated, and the pituitaries removed and homogenized. After extracting the protein content with 0.1 M HCl, the homogenates were centrifuged and supernatants stored at −20°C until assayed for LH, GH, and TSH by RIA. The protein content of the supernatants was determined by the method of Bradford (20), and hormone concentrations of the pituitaries were expressed as µg/mg protein. In experiment 2, four groups of five rats received the same treatment as in experiment 1, but the animals were sacrificed 1 week after the treatment, and LH, GH, TSH, and protein concentrations of the pituitaries were determined as described in experiment 1. This experiment was designed to investigate the effect of cytotoxic compounds AN-207 and AN-201 on the synthesis of LH, GH, and TSH at the time when the treated animals might show the greatest pituitary dysfunction as indicated by lowest BW.

**In vitro tests.** The superfused pituitary system was used for these experiments (21). One week after a single injection of 150 nmol/kg cytotoxic LH-RH analog AN-207 or 75 nmol/kg cytotoxic radical AN-201, two animals in both treated groups showing the greatest loss of BW, and no estrous cycle was used for the experiments in vitro, because it was thought that the greatest possible damage to pituitary functions, caused by the cytotoxic compounds, occurred at that time. Two rats treated with [D-Lys₆]LH-RH were used as controls. For the experiment performed at 3 weeks, the animals were selected randomly and tested at that time. In vitro experiments. The ovarian cycle of rats in the group treated with [D-Lys₆]LH-RH was also disturbed temporally, but regular cycles returned on days 4–5 in all 10 rats. Treatment with saline did not disturb the estrous cycle of the rats.


**Statistical Analysis of Data.** Results expressed as means ± SEM were evaluated by ANOVA; when the P value was <0.05, the analysis was completed using Duncan’s multiple range test. The superfusion data were analyzed with a computer program developed in our institute (21). Using this program we analyzed the peaks and calculated the amount of hormone secreted above the baseline.

**RESULTS**

**Body Weights.** Cytotoxic LH-RH analog AN-207 at a dose of 150 nmol/kg caused a significant 15% decrease in the BW of rats as compared with controls injected with [D-Lys₆]LH-RH or saline (P < 0.01), 1 week after the treatment. After the 1st week, the BW of the AN-207-treated animals increased gradually, and at the 2nd, 3rd, 4th, and 5th weeks relative losses of BW were 14%, 13%, 9%, and 8% (P < 0.05), respectively (Fig. 1). By the 6th and 7th weeks, BW of these rats did not differ significantly from the controls. Treatment with a 75 nmol/kg dose of cytotoxic radical AN-201 also caused significant 8%, 11%, and 12% decrease in the BW of rats at weeks 1, 2, and 3, respectively, as related to controls (P < 0.05, P < 0.05, and P < 0.01, respectively). The BW of these rats was similar to the controls by the 4th week. No significant differences in BW were found between the two groups treated with cytotoxic compounds at any time period (Fig. 1).

**Ovarian Cycles.** After a single injection of cytotoxic LH-RH analog AN-207, vaginal smears in 8 of 10 rats showed diestrus for 3–5 days. The estrous cycle returned 4–6 days after the treatment, and remained regular throughout the 7-week experiment. In 2 of 10 rats, the estrous phase of cycle did not appear by day 7 when they were sacrificed for in vitro experiment. Cytotoxic radical AN-201 did not alter the cycle in three rats and disturbed only the first cycle following the treatment in five animals. Estrous cycles of these five animals returned on days 5–7 and remained regular throughout the study. As in the group treated with AN-207, in 2 of 10 rats, the estrous phase did not return by the 7th day after treatment, when they were sacrificed for in vitro experiments. The ovarian cycle of rats in the group treated with [D-Lys₆]LH-RH was also disturbed temporally, but regular cycles returned on days 4–5 in all 10 rats. Treatment with saline did not disturb the estrous cycle of the rats.

**Pituitary Function Tested in vitro at Weeks 1 and 3.** The results of the in vitro study are shown in Table 1 and Fig. 2. LH release. One week after the in vivo treatment with AN-207, the net integrated LH released by the pituitary cells...
in response to 1 nM and 10 nM LH-RH was decreased by 63% as compared with the control value (Fig. 2a). Treatment with AN-201 caused a 57% decrease in the LH response. LH responses to the nonspecific membrane depolarizing KCl were also diminished by 52% for AN-207 and by 53% in the case of AN-201 (Fig. 2a). The total LH content of the cells from rats treated with cytotoxic hybrid AN-207 was reduced by 27% and that from AN-201-treated rats was lowered by 29%. Three weeks after the treatment, the receptor-mediated LH response of the cells of rats given AN-207 increased by 26% and that of the AN-201-treated animals augmented by 97%. Nonspecific (NSP) release of LH, GH, and TSH was increased by 21% for AN-207 and by 55% for AN-201 (Fig. 2c). The residual content of LH in the pituitary cells of AN-207-treated rats was not significantly different from that of the control rats (>5%), and it was reduced by 13% in the cells of the AN-201-treated rats.

GH release. One week after the treatment with cytotoxic hybrid AN-207 at a dose of 150 nmol/kg, there was only a 11% decrease in the GH-RH-induced GH release, and no significant change in the nonspecific GH release (<5%) (Fig. 2b) or the total GH content of the cells (<5%), as compared with control values. However, treatment with 75 nmol/kg of cytotoxic radical AN-201 suppressed the GH-RH-induced release of GH by 79%, the K+-induced nonspecific GH response by 69% (Fig. 2b), and lowered the residual content of GH in the cells by 51%. Three weeks after the treatment with the cytotoxic compounds, the GH responses of both treated groups returned to normal. At this time, differences of less than 10% were found in the receptor-mediated and the nonspecific GH responses of the cells between the treated and the control groups (Fig. 2d).

TSH release. Pituitaries of rats treated with cytotoxic LH-RH conjugate AN-207 at 150 nmol/kg dose, showed only a 12% decrease in the specific TSH response to TRH and a 11% diminution of the nonspecific TSH release by K+, at 1 week after treatment. The TSH content was not significantly changed in these cells, as compared with controls (Table 1). However, treatment with cytotoxic radical AN-201 at a 75 nmol/kg dose caused significantly greater decrease in TSH release than the cytotoxic conjugate, reducing the receptor-mediated TSH response of the pituitary cells by 67% and the nonspecific TSH release by 59%. At the end of the experiment, a 59% decrease in the residual TSH content was found in these cells, as compared with controls (Table 1). Three weeks after the treatment, no significant differences could be observed in the TSH responses (>10%) or in the residual TSH content of the cells (>10%) between the three groups (Table 1).

Table 1. LH, GH, and TSH responses in superfused rat pituitary cells to specific releasing hormone and nonspecific releasing challenges after an in vivo treatment with cytotoxic LH-RH analog (AN-207) and cytotoxic radical (AN-201)

<table>
<thead>
<tr>
<th>Groups</th>
<th>LH, ng</th>
<th>GH, μg</th>
<th>TSH, ng</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SP</td>
<td>NSP</td>
<td>Content</td>
</tr>
<tr>
<td>AN-207</td>
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<td>64.6</td>
<td>1740</td>
</tr>
<tr>
<td>AN-201</td>
<td>109</td>
<td>73.0</td>
<td>1681</td>
</tr>
<tr>
<td>Control*</td>
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<td>154</td>
<td>2371</td>
</tr>
<tr>
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<td>367</td>
<td>222</td>
<td>1826</td>
</tr>
<tr>
<td>AN-201</td>
<td>574</td>
<td>283</td>
<td>1564</td>
</tr>
<tr>
<td>Control*</td>
<td>291</td>
<td>183</td>
<td>1796</td>
</tr>
</tbody>
</table>

*Control rats were treated with the carrier [d-Lys⁶]LH-RH.

**Fig. 1.** BW of the rats treated with cytotoxic LH-RH analog AN-207, cytotoxic radical AN-201, and the carrier [d-Lys⁶]LH-RH (control). BW of rats given saline were similar to [d-Lys⁶]LH-RH control and are not shown. Data points represent mean ± SEM (n = 6–10). *, P < 0.05; **, P < 0.01 versus control.

**Table 1.** LH, GH, and TSH responses in superfused rat pituitary cells to specific releasing hormone and nonspecific releasing challenges after an in vivo treatment with cytotoxic LH-RH analog (AN-207) and cytotoxic radical (AN-201).
beseen in the time course of LH response between the treated and the control rats. Serum LH concentrations in rats treated with cytotoxic compounds were higher at 60 min than at 5 min after LH-RH challenge, while those of the control rats decreased between 5 and 60 min. LH levels of all animals returned to the basal values 180 min after the LH-RH injection (Fig. 3d). Six weeks after treatment, basal LH levels and LH responses to LH-RH were found to be normal in both groups treated with the cytotoxic compounds, compared with the control group (these data are not shown in Fig. 3).

**GH release.** Single injections of cytotoxic hybrid AN-207 or cytotoxic radical AN-201 to rats did not change the basal GH secretion or the GH-RH-stimulated GH secretion measured 2 weeks or 4 weeks after administration (Fig. 3b and c). GH responsiveness remained normal in both treated groups compared with controls at 6 weeks after treatment.

**TSH release.** The TSH secretory function of the pituitary was also unaffected by treatment with the cytotoxic compounds at all time period tested (Fig. 3 c and f).

**Pituitary LH, GH, and TSH Concentrations.** Table 2 shows the LH, GH, and TSH concentrations of pituitaries measured 1 and 7 weeks after a single i.v. administration of cytotoxic LH-RH analog AN-207, its cytotoxic radical AN-201, and the carrier [d-Lys⁶]LH-RH (control) to female rats. No significant differences could be found in the concentration of these pituitary hormones between the three groups at 1 or 7 weeks after treatment. These values were also similar to those found in rats injected with saline (data not shown).

**DISCUSSION**

Targeted cytotoxic LH-RH analogs have been developed in an endeavor to reduce the toxic side effects and increase the efficacy of antineoplastic agents by delivering them more selectively to tumor cells expressing receptors for LH-RH (1, 18). Because gonadotroph cells of pituitary possess high affinity receptors for LH-RH, it is important to determine whether these analogs would damage pituitary functions. It was previously shown that the cytotoxic LH-RH analog AN-207 used in this study fully preserves both the high binding affinity to LH-RH receptors and the powerful LH-releasing activity of the carrier molecule [d-Lys⁶]LH-RH, in rat pituitary cells (18). Because of the incorporation of the intensely potent 2-pyrrolino-DOX radical AN-201, the cytotoxic activity in vitro of AN-207 on MCF-7 human mammary carcinoma cell line possessing LH-RH receptors (22) was shown to be 500-1000 times higher than that of the hybrid AN-152 consisting of [d-Lys⁶]LH-RH linked to DOX (18). DOX is a bioreductive drug that is activated by intracellular enzymes to release the cytotoxic moiety.
intercalating agent that inhibits DNA synthesis (23, 24), and its effect on cell membranes as the first target of action was also reported (25, 26). Because the cycle of the pituitary cells is rather long, the damaging effect of DOX and its derivatives may be detectable by an impairment of cell functions other than mitotic division. The results of this study show that AN-207 caused selective damage of the pituitary gonadotroph cell function 1 week after the treatment, diminishing the AN-207 caused selective damage of the pituitary gonadotroph than mitotic division. The results of this study show that damage to pituitary cells could be in any case alleviated by appropriate replacement therapy.

Cytotoxic radical AN-201 caused a nonselective damage to the pituitary, as shown by a similar reduction in the receptor-mediated release of LH, GH, and TSH as well as in the nonspecific release of these hormones. A similar selectivity of damage was shown in a previous study in vitro using pharmacological doses of early cytotoxic LH-RH analogs much less potent than AN-207 (27). The total immunoreactive hormone concentration of the pituitary was found to be essentially similar in the animals treated with AN-207 and AN-201 and control animals in vivo at 1 week after treatment. In view of this finding, it is likely that decreases in the residual hormone content in the cells of the superfusion study were caused by the loss of the injured cells during the preparative procedure (digestion with collagenase, mechanical dispersion) rather than a deficiency in the hormone synthesis. It has to be noted that the rats used for this superfusion experiment had the lowest BW due to toxicity, which could reflect the greatest dysfunction of pituitary cells. Our observation that the highest deprivation of hormone-releasing functions was observed in the GH (74%) and the smallest in the LH release (57%) is in agreement with the conclusion of a study in humans that GH cells are the most vulnerable of all the pituitary cells to irradiation (28).

The results of the present study also show that the toxic effect of cytotoxic LH-RH analog AN-207 and its cytotoxic radical AN-201 on the pituitary function is reversible. Two weeks after the treatment with these cytotoxic compounds, the receptor-mediated LH-releasing function of the pituitary of rats treated with AN-207 showed a complete recovery. At this time period, a slight decrease in LH response to LH-RH could still be found in the AN-201-treated rats. At 3 weeks following the treatment, a complete recovery as well as an increase of the LH secretory response was observed in vitro in the cells of both treated groups. Similar observations were made in recent studies in men, reporting that the LH response to an LH-RH test was exaggerated and the amplitude of LH pulses was also significantly elevated following chemotherapy with cytotoxic drugs (28). A greater responsiveness of the LH cells to LH-RH was considered to be a compensatory process in men after an impairment of the Leydig cell function due to chemotherapy.

![Figure 3](image-url)
(28). Such a compensatory process with an increase in LH response may have also occurred in our study in female rats. Although no significant differences were found in the LH-RH induced LH responses between treated and the control groups at 4 weeks after treatment, somewhat higher and more prolonged LH release could be seen in the groups treated with the cytotoxic compounds, signifying that the compensatory process was still in effect.

Another important inference from this work is that the toxic effect of the cytotoxic radical is greater than that of the cytotoxic conjugate. In spite of the fact that the i.v. dose of AN-207 was 2 times higher on molar basis than that of AN-201, no significant difference was found in the BW between the two treated groups. Similar results were obtained in our previous study, in which we administered these cytotoxic compounds by i.p. route to male rats (19) and in a preliminary study in female rats.

In conclusion, the cytotoxic LH-RH analog AN-207 is highly selective for the cells containing LH-RH receptors, and less toxic to other cells, while its cytotoxic radical AN-201 nonselectively injures various cells of the pituitary. The toxic effects of both cytotoxic compounds on the pituitary are completely reversible. Because of its high selectivity and reduced toxicity AN-207 should be more efficacious for treatment of tumors which possess receptors for LH-RH than its cytotoxic radical 2-pyrrolino-DOXAN-201.

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