Combination of a long-acting delivery system for luteinizing hormone-releasing hormone agonist with Novantrone chemotherapy: Increased efficacy in the rat prostate cancer model

(controlled release of peptide hormones/antineoplastic agents/combined therapy)

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ABSTRACT The combination of hormonal treatment based on a long-acting delivery system for the agonist [6-D-tryptophan]luteinizing hormone-releasing hormone ([D-Trp6]-LH-RH) with the chemotherapeutic agent Novantrone (mitoxantrone dihydrochloride) was studied in the Dunning R3327H rat prostate cancer model. Microcapsules of [D-Trp6]-LH-RH formulated from poly(DL-lactide-co-glycolide) and calculated to release a controlled dose of 25 μg/day were injected intramuscularly once a month. Novantrone (0.25 mg/kg) was injected intravenously once every 3 weeks. Three separate experiments were carried out. When the therapy was started 45 days after transplantation and continued for 70 days, tumor volume in the presence of the microcapsules (966 ± 219 mm³) or Novantrone (3606 ± 785 mm³) given alone was significantly decreased compared to controls (14,476 ± 3045 mm³). However, the combination of microcapsules and Novantrone caused a greater inhibition of tumor growth (189 ± 31 mm³) than the single agents. Similar effects were seen when the percent increase in tumor volume was examined. Tumor volume increased 10,527 ± 1803% for the control group. The inhibition of growth caused by the [D-Trp6]-LH-RH microcapsules alone (672 ± 153% increase in volume) was again greater than that caused by Novantrone alone (2722 ± 421% increase). The combination of the two agents was again the most effective, resulting in an increase in tumor volume of only 105 ± 29%. Control tumors weighed 30.0 ± 6.5 g. Tumor weights were much less in the groups treated with either microcapsules (3.28 ± 0.69 g) or Novantrone (19.53 ± 3.3 g) alone. The lowest tumor weights after 70 days of treatment were obtained in the group that received the combination of [D-Trp6]-LH-RH microcapsules and Novantrone (1.02 ± 0.2 g). Testes and ventral prostate weights were significantly diminished by the administration of microcapsules of [D-Trp6]-LH-RH alone or in combination with Novantrone. In both of these groups, luteinizing hormone and prolactin levels were reduced and serum testosterone was suppressed to undetectable levels. Similar results were obtained in two other experiments in which the duration of treatment was 60 or 105 days. These results suggest that the overall response could reflect the inhibition of proliferation of hormone-independent cancer cells by Novantrone in addition to the suppressive effect of [D-Trp6]-LH-RH on the growth of androgen-dependent tumor cells. The administration of Novantrone in combination with microcapsules of [D-Trp6]-LH-RH might produce a better clinical response than LH-RH analog alone in patients with advanced prostate carcinoma.

We have recently developed long-acting delivery systems for the 6-D-tryptophan analog of luteinizing hormone-releasing hormone ([D-Trp6]LH-RH) based on microcapsule formulation of this analog in the biodegradable polymer poly(DL-lactide-co-glycolide) (1). Once-a-month intramuscular administration of microcapsules of [D-Trp6]-LH-RH to rats bearing androgen-dependent Dunning R3327H prostate adenocarcinoma markedly inhibited tumor growth and suppressed testosterone serum levels more effectively than daily subcutaneous injections of equivalent, or larger, doses of unencapsulated analog (1). The clinical therapeutic efficacy of these long-acting microcapsule preparations in patients with prostate cancer was also recently established by Roger et al. (2) and Parmar et al. (3). This approach, which makes the treatment more convenient and efficacious as compared with daily administration and ensures patient compliance, could become the method of choice for the endocrine treatment of advanced prostate carcinoma. Nevertheless, the duration of remissions may be limited, as hormonal manipulations do not prevent the ultimate growth of hormone-independent cells (4–9).

It has been demonstrated that carcinomas of the prostate are heterogeneous and contain both hormone-dependent and hormone-independent cells (1, 7, 10). After androgen deprivation, most of the hormone-dependent cells stop proliferating and die, but hormone-independent cells originally present in the tumor, by a clonal selection process, eventually repopulate the tumor (7, 9). As the prostate cancer progresses to a hormone-independent state, it becomes unresponsive to androgen-deprivation therapy (7). Chemotherapy inhibits the proliferation of androgen-independent cells. It is thus possible that the use of a combination of chemotherapeutic and hormonal approaches would prevent or delay a condition whereby hormone-insensitive cells present in the originally endocrine-dependent tumor become the predominant component (4–11). We have previously evaluated the combination of microcapsules of [D-Trp6]-LH-RH with cyclophosphamide (Cytoxan), an established antineoplastic drug commonly used for chemotherapy in prostate cancer. In the Dunning R3327H prostate cancer, the combination of Cytoxan with the microcapsules was much more effective than the single agents in reducing tumor weights (12).

In this study we used Novantrone (mitoxantrone dihydrochloride) (13–16), which is a modern synthetic chemotherapeutic agent with a putative mode of action similar to that of the anthracycline antibiotic Adriamycin but which is considerably less toxic. Novantrone was selected on the basis of its activity in a wide range of experimental animal tumors and its inhibitory effects on both proliferating and nonproliferating tumor cell lines.

Abbreviations: LH-RH, luteinizing hormone-releasing hormone; [D-Trp6]-LH-RH, 6-D-tryptophan analog of LH-RH; LH, luteinizing hormone.

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cells (13–16). The aim of this investigation was to determine whether the combination of hormonal therapy using microcapsules of [d-Trp⁶]LH-RH and chemotherapy based on Novantrone would enhance the inhibition of tumor growth in the Dunning R3327H prostate cancer model.

MATERIALS AND METHODS

Male (Copenhagen × Fisher)F₁ rats bearing the androgen-dependent well-differentiated R3327H Dunning rat prostate adenocarcinoma were provided by Norman Altman (Papanicolaou Cancer Research Inst., Miami, FL). Tumors were measured weekly by using microliterpens and volume was calculated by using the formula length × width × height × 0.5236 (17). In the first experiment, the treatment was initiated 60 days after transplantation at a time when tumors were well developed (tumor volume, 2000 mm³) and lasted 60 days. In the second study, treatment was started 135 days after transplantation when tumors were detectable, but still unmeasurable, and was carried on for 105 days. In the third experiment, the therapy was initiated 45 days after tumor inoculation and was continued for 70 days.

Novantrone (mitoxantrone dihydrochloride) (1,4-dihydropyrene, 5,8-bis-[2-[2-hydroxyethylamino]ethyl]-9,10-anthracenedione dihydrochloride) (American Cyanamid, Pearl River, NY) was diluted with 0.9% saline and injected intravenously at a dose of 0.25 mg/kg of body weight once every 3 weeks.

Microcapsules of [d-Trp⁶]LH-RH were prepared by P. Orsolini at Cytotech (Martigny, Switzerland) and kindly supplied by R. Y. Mauvernay (Debiopharm, Lausanne, Switzerland). The product consisted of [d-Trp⁶]LH-RH (2.1%, wt/wt) distributed within a polymeric matrix of 53:47 (mol%) poly(β-t-lactide-co-glycolide) (97.9%, wt/wt). The microcapsules in aliquots of 33 mg, calculated to release a dose of about 25 μg/day for 30 days, were suspended in disposable syringes in 0.7 ml of injection vehicle containing 2% CM-cellulose and 1% Tween 20 in water. This suspension was thoroughly mixed and injected through an 18-gauge needle deep into the thigh muscle of rats (1, 18). The administration was repeated at monthly intervals. The rats (9–12 per group) were divided into four groups. Group 1, the control, was injected with vehicle only and group 2, with the microcapsules. Group 3 received Novantrone and group 4 was treated with a combination of microcapsules and Novantrone.

After the treatment period, the rats were sacrificed by decapitation. Trunk blood was collected and serum was separated for further analysis. Tumors and various organs were removed, cleaned, and carefully weighed. Serum concentrations of luteinizing hormone (LH), prolactin, and testosterone were measured by using radioimmunoassays as previously described (1, 18, 19). All data are described as the mean ± SEM. Statistical analyses were performed by using a computer program and Duncan’s new multiple-range test (20) or Student’s t test.

Fragments of tumors were frozen in liquid nitrogen for receptor measurements or fixed in Bouin’s fluid for detailed histological examination, embedded in paraffin, and stained with hematoxylin/eosin, Masson’s trichromatic stain, and periodic acid/Schiff reagent.

RESULTS

All three experiments in which Novantrone treatment was combined with [d-Trp⁶]LH-RH microcapsules, carried out over a period of nearly 2 years, produced similar results. Therefore, only the results of experiment 3, in which the tumors showed the most rapid growth after inoculation, will be described in detail. Tumor volume was significantly reduced by the administration of microcapsules (966 ± 219 mm³; P < 0.01) or Novantrone (3606 ± 785 mm³; P < 0.01) as compared to the control group (14,476 ± 3045 mm³) (Fig. 1). Throughout the treatment period of 70 days, the microcapsules of [d-Trp⁶]LH-RH reduced tumor volume more than Novantrone given alone. However, the combination of these two agents caused a greater inhibition of tumor growth (189 ± 31 mm³) than the microcapsules or Novantrone administered alone (P < 0.01 vs. control; P < 0.005 vs. microcapsules by Student’s t test) (Fig. 1). Similar results were obtained in experiment 1, in which the tumors grew at the same rate, and in experiment 2, in which the tumors showed a slower growth than in experiment 3, the untreated controls reaching a volume of 7306 ± 3976 mm³. The effects of the administration of the two agents on the percent change in tumor volume during the period of the experiment are shown in Fig. 2. As measured by this parameter, the inhibition induced by the [d-Trp⁶]LH-RH microcapsules (672 ± 153% compared to the control of 10,527 ± 1803%; P < 0.01 vs. control) was again much greater than that of Novantrone (2722 ± 421%; P < 0.01 vs. control). The combination of the two agents (105 ± 29%; P < 0.01 vs. control) was again clearly the most effective and resulted in the greatest inhibition of the percent increase in tumor volume (Fig. 2). This inhibition was significantly greater than that obtained with microcapsules alone (P < 0.005 by Student’s t test). Similar findings were recorded in experiments 1 and 2.

These conclusions based on tumor volume were confirmed by determination of tumor weights on autopsy, when the control tumors were found to weigh 30.0 ± 6.5 g. The lowest tumor weights after 70 days of treatment were obtained in the group that received the combination of [d-Trp⁶]LH-RH and Novantrone (Table 1): 1.02 ± 0.20 g (P < 0.01 vs. control by Duncan’s test and P < 0.005 vs. group given microcapsules by Student’s t test). Lower tumor weights were also observed in the groups treated with either microcapsules (3.28 ± 0.7 g) or Novantrone alone (19.53 ± 3.3 g), and the microcapsules were more effective than Novantrone. Similar results were obtained in experiment 2 with slow-growing tumor: the untreated control tumors weighed 10 ± 4.4 g, whereas the group given the combination of microcapsules and Novantrone had small tumors that weighed only 0.06 ± 0.01 g (P < 0.005 vs. microcapsules alone by Student’s t test), the combination virtually stopping tumor growth. In experiment
1, tumor weights were also much less in the group that received the combination of [D-Trp6]LH-RH and Novantrone (P < 0.05 vs. controls). Low tumor weights were observed, too, in the groups treated with either microcapsules or Novantrone alone, but because the treatment was started when tumors were well developed, the inhibition of the increase in tumor weights with single agents was not significant.

It can be seen in Table 1 that testes and ventral prostate weights were significantly diminished by microcapsules alone or in combination. Similar results were observed in experiments 1 and 2. Serum testosterone, LH, and prolactin were significantly reduced by administration of microcapsules of [D-Trp6]LH-RH alone or in combination with Novantrone (Table 2).

Every tumor was examined histologically. All tumors were well-differentiated adenocarcinomas (Fig. 3 Left), but in the groups treated with microcapsules and combination of microcapsules and Novantrone, the number of the cells decreased and the glandular structures (adenocarcinomas) showed considerable atrophic signs as compared to the control group (Fig. 3 Right). In the group treated with Novantrone alone, fewer alterations occurred. A very important finding is the increase of the connective tissue in the group treated with the combination of microcapsules and Novantrone (Fig. 3 Right). This increase was smaller in the group treated with microcapsules and almost nonexistent in the group treated with Novantrone. These histological results suggest that all three therapies inhibit the growth of the tumoral cells, and these cells are replaced by connective tissue.

**DISCUSSION**

This work was the continuation of our investigations on the combination of hormonal treatment based on LH-RH analogs with chemotherapeutic agents in an attempt to enhance therapeutic efficacy. We investigated the synergistic effect of modern hormonal therapy utilizing LH-RH agonists combined with the antineoplastic drug Novantrone in rats bearing hormone-dependent Dunning R3327H prostatic adenocarcinoma at various stages of development.


Clinical efficacy of [D-Trp6]LH-RH and at least three other related agonists of LH-RH in the palliative treatment of prostatic carcinoma has been clearly demonstrated by several different groups of investigators (2, 3, 22-34). The therapy based on agonists of LH-RH avoids the cardiovascular and mammotrophic side effects of estrogens and the psychological impact of orchiectomy (22-36). It has been suggested that treatment with [D-Trp6]LH-RH produces a higher rate of response than transcapsular orchiectomy (35). The use of LH-RH agonists may be the method of choice for the treatment of prostate cancer (3, 22-36). Clinical trials with microcapsules of [D-Trp6]LH-RH and implants of LH-RH agonist 118630 (Zoladex) in patients with prostate cancer attest to their high efficacy, compliance, and convenience as compared to daily administration of the unencapsulated agonist (3, 36). The microcapsules of [D-Trp6]LH-RH have

![Figure 2](image-url)

**Table 1. Effects of [D-Trp6]LH-RH microcapsules, Novantrone, and their combination on body, organ, and tumor weight in rats with Dunning R3327H prostate tumors**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body, g</th>
<th>P vs. control</th>
<th>Pituitary, mg</th>
<th>P vs. control</th>
<th>Adrenal, mg</th>
<th>P vs. control</th>
<th>Testes, g</th>
<th>P vs. control</th>
<th>Ventral prostate, mg</th>
<th>P vs. control</th>
<th>Tumor, g</th>
<th>P vs. control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>380 ± 8</td>
<td>—</td>
<td>7.62 ± 0.2</td>
<td>—</td>
<td>53.11 ± 1.19</td>
<td>—</td>
<td>2.68 ± 0.08</td>
<td>—</td>
<td>341 ± 11</td>
<td>—</td>
<td>30.01 ± 6.53</td>
<td>—</td>
</tr>
<tr>
<td>[D-Trp6]LH-RH</td>
<td>344 ± 6</td>
<td>&lt;0.01</td>
<td>6.77 ± 0.2</td>
<td>&lt;0.05</td>
<td>46.74 ± 1.89</td>
<td>&lt;0.05</td>
<td>0.49 ± 0.02</td>
<td>&lt;0.01</td>
<td>63 ± 6</td>
<td>&lt;0.01</td>
<td>3.28 ± 0.69</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>microcapsules</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Novantrone</td>
<td>365 ± 6</td>
<td>NS</td>
<td>8.13 ± 0.2</td>
<td>NS</td>
<td>51.17 ± 1.91</td>
<td>NS</td>
<td>2.74 ± 0.04</td>
<td>NS</td>
<td>301 ± 14</td>
<td>NS</td>
<td>19.53 ± 3.29</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>+ microcapsules</td>
<td>328 ± 6</td>
<td>&lt;0.01</td>
<td>6.51 ± 0.21</td>
<td>&lt;0.01</td>
<td>48.70 ± 1.22</td>
<td>NS</td>
<td>0.44 ± 0.02</td>
<td>&lt;0.01</td>
<td>30 ± 3</td>
<td>&lt;0.01</td>
<td>1.02 ± 0.19</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Microcapsules released 25 μg/day and were administered every 30 days for 70 days. Novantrone was 0.25 mg/kg of body weight i.e. every 3 weeks for 70 days. The therapy was initiated 45 days after tumor transplantation and continued for 70 days. The results are the means ± SEM of groups of 10–12 rats and probabilities were calculated by Duncan’s new multiple range test. NS, not significant.

*P < 0.001 vs. combination by the Student’s t test.

†P < 0.005 vs. microcapsules by Student’s t test.
been used successfully for the treatment of women with endometriosis (37) and children with precocious puberty (38). However, the duration of response and the median survival time in patients with prostate cancer cannot yet be estimated.

It is well established for other hormonal methods of treatment that, in most patients with prostate cancer, the duration of remission may be limited and a relapse eventually occurs (5–11). Recently, one patient relapsed after 18 months of objective and subjective remission in response to [D-Trp⁶]LH-RH treatment, in spite of continued blockade of pituitary-gonadal axis, and had to be treated by chemotherapy with cyclophosphamide (34). This relapse is attributed to a selective proliferation of clones of androgen-independent cancer cells that preexisted within the heterogeneous prostate tumor (7–9). While hormone-dependent tumor cells stop growing after androgen ablation, the testosterone-insensitive cells are able to proliferate and eventually become predominant, although they may have represented initially only a small fraction of the starting tumor (7, 9). The response rates in advanced prostatic carcinoma that are obtained with chemotherapy alone are generally low (10, 11). The aim of combining hormonal therapy with chemotherapy is to prevent the proliferation of both hormone-dependent and hormone-independent cells. The use of both approaches, endocrine and chemotherapeutic, may increase the rate of response and its duration (5–11, 35).

Previous study from this laboratory in rats bearing Dunning R3327H prostate tumors (1) has shown that when the combination therapy with microcapsules of [D-Trp⁶]LH-RH and the alkylating agent cyclophosphamide was initiated early in the progression of tumor development, then tumor growth was inhibited more effectively than when single agents were used (12). In the present experiments on the evaluation of the combination of hormonal therapy based on [D-Trp⁶]LH-RH with chemotherapy in prostate cancer we have used Novantrone (mitoxantrone dihydrochloride), an anthracycline with some structural similarities to Adriamycin but without an amino-sugar moiety (13–16). Novantrone was reported to have equal or superior antitumor efficacy as compared to Adriamycin but reduced or no cardiotoxicity (13). Adriamycin binds to DNA and inhibits RNA and DNA synthesis. Studies at the cellular level have shown that Novantrone has inhibitory effects on both proliferating (dividing) and nonproliferating (nondividing) cells (14, 15). Thus, the cell-killing ability of this drug is not cell-cycle specific. Novantrone has a wide spectrum of activity against experimental murine tumors and shows synergism with cisplatin, cyclophosphamide, and other antineoplastic agents in colon and mammary adenocarcinoma. Novantrone was reported to induce a significant response in patients with breast cancer (16). Since Adriamycin produces an objective response rate of about 25% in patients with metastatic prostate carcinoma, and Novantrone is much less toxic than Adriamycin, we have decided to evaluate its effect in the Dunning model of prostate adenocarcinoma.

Chemotherapy is generally used in patients with advanced prostate cancer after they have become unresponsive to hormonal treatment (5, 6, 11). However, Murphy et al. (8) and Mukamel et al. (5) used chemotherapeutic agents in combination with hormonal therapy in patients with metastatic prostate carcinoma soon after the diagnosis was made. A simultaneous or sequential administration of hormonal...

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Serum LH, pg/ml</th>
<th>P vs. control</th>
<th>Serum prolactin, ng/ml</th>
<th>P vs. control</th>
<th>Serum testosterone, ng/ml</th>
<th>P vs. control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>714 ± 61</td>
<td>—</td>
<td>15.04 ± 2.3</td>
<td>—</td>
<td>1.98 ± 0.64</td>
<td>—</td>
</tr>
<tr>
<td>[D-Trp⁶]LH-RH microcapsules</td>
<td>228 ± 15</td>
<td>&lt;0.01</td>
<td>8.5 ± 0.86</td>
<td>&lt;0.01</td>
<td>ND</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Novantrone</td>
<td>415 ± 54</td>
<td>&lt;0.01</td>
<td>6.5 ± 0.81</td>
<td>&lt;0.01</td>
<td>1.46 ± 0.36</td>
<td>NS</td>
</tr>
<tr>
<td>Novantrone + microcapsules</td>
<td>309 ± 25</td>
<td>&lt;0.01</td>
<td>7.5 ± 0.83</td>
<td>&lt;0.01</td>
<td>ND</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

Microcapsules released 25 µg/day and were administered every 30 days for 70 days. Novantrone was 0.25 mg/kg of body weight i.v. every 3 weeks for 70 days. The therapy was initiated 45 days after tumor transplantation and continued for 70 days. The results are the means ± SEM of groups of 10–12 rats and probabilities were calculated by Duncan’s new multiple range test, except where noted. NS, not significant; ND, not detectable by radioimmunoassay.

*Calculated by Student’s t test.

In FIG. 3. Histological sections of prostate tumors. (Masson’s trichrome stain.) (Left) Tumor from the control (untreated) group, showing a well-differentiated adenocarcinoma. (×70.) (Right) Tumor from the group treated with the combination of microcapsules of [D-Trp⁶]LH-RH and Novantrone. A decrease in the number of cells and an increase in interstitial connective tissue can be noted. (×285.)
treatment based on microcapsules of the LH-RH agonist and chemotherapy could be superior to either modality alone, since a single approach affects only a portion of the tumor population (7).

The timing of such combined therapy may be of great importance, as suggested by studies in experimental rat models (7) and in patients (5, 6). Various findings reported previously indicate that combined therapy should be initiated soon after diagnosis is established (5, 12). In addition, the response of cancer cells to chemotherapy might be potentiated by an early and transitory increase in testosterone levels after initial administration of LH-RH agonists. Thus, a hormonal treatment could stimulate cell division, recruit the cells into the cycle, partially synchronize the phase of cell growth, and significantly enhance the cells’ sensitivity to some cytostatics. The fibroblastic proliferation observed in the histological study may indicate a favorable response to the therapy. Consequently, our present view is that combined therapy should be initiated, with chemotherapy being given several hours after the first administration of LH-RH agonists (35).

The present results demonstrate the effectiveness of hormonal treatment based on microcapsules of [D-Trp⁶]-LH-RH combined with Novantrone chemotherapy in suppressing the growth of Dunning R3327H prostate tumors in rats. Our results suggest that the combination of Novantrone with microcapsules of [D-Trp⁶]-LH-RH might lead to a better therapeutic response than the treatment with LH-RH analog alone in patients with prostate carcinoma.

We are grateful to Dr. R. Y. Mauvernay (Debiopharm, Lausanne, Switzerland) and Dr. P. Orsolini (Cytoetch, Martigny, Switzerland) for a generous supply of microcapsules of [D-Trp⁶]-LH-RH and to Dr. D. K. McClintock and Dr. C. E. Traitor (American Cyanamid, Pearl River, NY) for the supply of Novantrone and helpful suggestions. We thank the National Hormone and Pituitary Program for gifts of materials used in radioimmunoassays. This work was supported by National Institutes of Health Grant AM-07647 and CA-40003 and the Veterans Administration Research Service.