Suppression of meiosis of male germ cells by an antagonist of luteinizing hormone-releasing hormone

(analogs of luteinizing hormone-releasing hormone/inhibition of spermatogenesis)

Bela Szende†, Tommie W. Redding‡, and Andrew V. Schally*††

*Endocrine, Polypeptide and Cancer Institute, †Veterans Administration Medical Center, New Orleans, LA 70146; and ‡Section of Experimental Medicine, Department of Medicine, Tulane University School of Medicine, New Orleans, LA 70112

Contributed by Andrew V. Schally, November 20, 1989

ABSTRACT Male nude mice were implanted with osmotic minipumps releasing 50 μg of a potent antagonist of luteinizing hormone-releasing hormone (LH-RH) per day [N-Ac-[d-Nal(2)]², d-Phe(pCI)]², d-Pal(3)]³, d-Cit⁶, d-Ala¹⁰[LH-RH] (SB-75) [Nal(2), 3-(naphthyl)alanine; Phe(pCI), 4-chlorophenylalanine; Pal(3), 3-(3-pyridyl)alanine; Cit, citrulline], or they were treated with s.c. injections of SB-75 (25 μg twice a day). Another group of nude mice received an injection of microcapsules of the agonist [d-Trp⁶]LH-RH liberating 25 μg/day. One month after the initiation of treatment, the testicular weights were significantly reduced and the blood testosterone values were at castration levels in all treated groups. Histologically, only the testicles of the mice treated with SB-75 released from minipumps showed a significant decrease of meiosis. The most advanced forms of germ cells were spermatogonia in 26%, spermatocytes in 17%, and round spermatids in 35% of the seminiferous tubules. Only 22% of the tubules contained elongated spermatids. The suppression of meiotic activity by this modern LH-RH antagonist can possibly be used for the development of methods for male contraception and for the protection of germ cells against the damage caused by cytotoxic drugs and x-radiation.

The possible application of analogs of luteinizing hormone-releasing hormone (LH-RH) for development of new contraceptive methods has been investigated for more than a decade (1–4). Potent LH-RH agonists have been developed and used clinically in the areas of cancer and gynecology (2, 3). However, the suitability of LH-RH agonists for male contraception has been questioned because of the induction of impotence when LH-RH agonists alone are used or because of the risks when chronic testosterone supplementation is given to overcome the adverse effects of the LH-RH agonist treatment on libido and potency (2, 3, 5). LH-RH antagonists also exert antifertility effects in female and male rats (6–9) as well as in monkeys (10–12). We showed that pretreatment of rats with one of these antagonists, N-Ac-[d-Phe(pCI)]², d-Trp², d-Arg³, d-Ala¹⁰[LH-RH] [Phe(pCI), 4-chlorophenylalanine] exerted a protective effect against x-radiation or chemotherapy-induced testicular damage because of temporary arrest of germ cell proliferation at the time of irradiation or chemotherapy (13, 14). However, this class of antagonists with d-Arg in position 6 produced transient edema due to liberation of histamine (15).

To eliminate the undesirable edematosogen effects seen with these antagonists, modern antagonists were synthesized in our laboratory containing the ureidoalanyl amino acid d-citrulline (Cit) in position 6 (16). Some analogs such as N-Ac-[d-Nal(2)]², d-Phe(pCI)]², d-Pal(3)]³, d-Cit⁶, d-Ala¹⁰[LH-RH] (SB-75) also had d-3-(3-pyridyl)alanine [Pal(3)] in place of d-Trp in position 3 [Nal(2), 3-(naphthyl)alanine]. These highly potent antagonists administered in small doses blocked ovulation in cycling rats and suppressed LH levels in ovariectomized rats (16). Recently, we showed that antagonist SB-75 in a total dose of 75–600 μg s.c. or i.m. suppressed gonadotropin levels in human beings. These doses are much smaller than those used for other antagonists (15), indicating very high activity of SB-75. This class of peptides was completely free of toxic side effects even at high doses.

In view of the extensive use of the nude mouse model for the investigation of transplanted human cancers, we also demonstrated a strong suppression of the pituitary–gonadal axis in nude mice by continuous administration of LH-RH antagonists. In this study, we report the effect of the LH-RH antagonist SB-75 on the testicular histology in nude mice.

MATERIALS AND METHODS

Athymic nude (nu/nu) male mice, ~6 weeks old on arrival, were obtained from the National Cancer Institute. Mice were housed in sterile plastic cages and were placed in a continuous laminar air flow hood. Commercially available pelleted diet, bedding, and water were sterilized and all manipulations of mice were carried out under sterile conditions. Ten animals were used per experimental group.

Since microcapsule formulation of the antagonist SB-75 was not available at the time these experiments were conducted, SB-75 was administered to mice by Alzet miniosmotic pumps (no. 2002, Alzet, Palo Alto, CA). These implantable minipumps delivered the LH-RH antagonist in a dose of 50 μg/day dissolved in 50% (vol/vol) propylene glycol in water, at a pumping rate of 0.5 μl/hr for a duration of 2 weeks. The minipumps were implanted s.c. in the dorsal area of mice under Metofane anesthesia by aseptic techniques. After 2 weeks, the spent pumps were removed and new filled pumps were reimplanted. Mice were monitored carefully for infection during the course of the experiment. Those mice showing signs of infections were removed from the experiment. A group of 10 male nude mice was treated by daily s.c. injections of 25 μg per animal of SB-75 twice daily. Another group of 10 animals received a single subcutaneous injection of the microcapsules of LH-RH agonist [d-Trp⁶]LH-RH in poly(DL-lactide-coglycolide) (Cytotech, Martigny, Switzerland) designed to maintain a continuous liberation of 25 μg/day for 30 days from an aliquot of 36 mg.

After 30 days of treatment, the mice were sacrificed by decapitation and trunk blood was collected and serum was separated for further analyses. Body weights were recorded

Abbreviation: LH-RH, luteinizing hormone-releasing hormone.


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and various organs were removed, cleaned, and carefully weighed. The testicles were fixed in Zamboni's fixative and embedded in paraplast. Six-micrometer-thick sections were cut and stained with hematoxylin and eosin. Testicular histology was studied to classify seminiferous tubules according to the presence of the most advanced germ cell (i.e., elongated and round spermatids, spermatocytes, and spermatogonia). A total of 100 tubules were examined in each animal and the results were evaluated statistically.

Serum levels of LH and testosterone were measured by radioimmunoassays as described (17). Statistical analyses were performed by using a computer-assisted program of Duncan's new multiple range test (18).

RESULTS

There were no significant changes in body weight in the treatment groups when compared to control mice. However, testes weights were significantly reduced by >40% in mice treated with antagonist SB-75 administered by minipumps (Table 1). In male nude mice injected with 25 μg twice daily of the antagonist SB-75, testes weights fell by only 18%. Administration of SB-75 either by minipump or by daily injections significantly decreased ventral prostate and seminal vesicle weights as shown in Table 1. The antagonist SB-75 significantly inhibited the levels of both LH and testosterone when delivered by minipumps as compared to control mice. Daily s.c. injections of 25 μg twice daily of SB-75 also significantly reduced LH by 82% and testosterone levels by 78% (Table 1).

Table 1. Effect of LH-RH antagonist SB-75 on the weight of testes, ventral prostate, and seminal vesicles and on the serum levels of LH and testosterone in male nude mice after 30 days of treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Testes, mg</th>
<th>Ventral prostate, mg</th>
<th>Seminal vesicles, mg</th>
<th>Serum LH, pg/ml</th>
<th>Serum testosterone, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>221 ± 3.4</td>
<td>12 ± 1.3</td>
<td>157 ± 9.8</td>
<td>794 ± 61</td>
<td>0.57 ± 0.13</td>
</tr>
<tr>
<td>SB-75 minipump (50 μg/day)</td>
<td>131 ± 7.0*</td>
<td>2 ± 0.5*</td>
<td>36 ± 5.4*</td>
<td>220 ± 46*</td>
<td>0.04 ± 0.013*</td>
</tr>
<tr>
<td>SB-75 s.c. (25 μg twice per day)</td>
<td>180 ± 9.2†</td>
<td>3 ± 0.5*</td>
<td>13 ± 1.2*</td>
<td>153 ± 45*</td>
<td>0.13 ± 0.011*</td>
</tr>
</tbody>
</table>

All values are means ± SE.

*P < 0.01 (Duncan's new multiple range test).
†P < 0.05.

Treatment with [p-Trp]$^6$LH-RH microcapsules decreased testes weight by 27% and reduced ventral prostate and seminal vesicle weights by 74% and 79%, respectively. Serum levels of LH and testosterone were decreased by 62% and 95%, respectively (data not shown).

Fig. 1 shows the median frequency of seminiferous tubules containing different germ cell forms as the most advanced germ cell in controls and in animals treated with SB-75 in osmotic minipumps. Elongated spermatids were present in 99.7% of the seminiferous tubules of the control mice. Only 0.3% of the tubules of the controls had round spermatids as the most advanced germ cell. Twenty-six percent of the seminiferous tubules of the mice treated with SB-75 in the form of minipumps contained only spermatogonia (Fig. 2); 17% contained spermatogonia and spermatocytes; 35% contained spermatogonia, spermatocytes, and round spermatids. Only 22% of the seminiferous tubules of the treated mice appeared to contain elongated spermatids. The suppression of spermatogenesis was highly significant (P < 0.01) when compared to the controls. The Leydig cells of the treated mice were normal. No histologically detectable inhibition of spermatogenesis was found in the group treated with daily injections of SB-75 or with microcapsules of [p-Trp]$^6$LH-RH. This means that 97–98% of the seminiferous tubules of the testicles of the mice in these groups contained elongated spermatids.

DISCUSSION

Our results indicate that a continuous release of the LH-RH antagonist SB-75 from the implantable osmotic minipumps can suppress significantly the spermatogenesis of nude mice.

![Fig. 1. Effect of antagonist SB-75 (released from osmotic minipumps) on the spermatogenesis of nude mice. Bars show the percent of seminiferous tubules in which spermatogonia (SGO), spermatocytes (SC), round spermatids (RS), and elongated spermatids (ES) are the most advanced forms of germ cells. Values are means ± SE. Statistical measurements were done using Duncan's test. All values for animals treated with SB-75 differ significantly from the control (P < 0.01).](image-url)
The presence of spermatogonia in all the seminiferous tubules of the mice treated with the minipumps releasing SB-75 points to the fact that no total suppression of spermatogenesis occurred and suggests that this suppression is most likely reversible. The suppression is shown by the inhibition of meiosis in a high percentage of the seminiferous tubules. The duration and reversibility of this effect will be examined in our future experiments. It is of major importance that daily injections of SB-75 did not cause the histological alterations in the seminiferous tubules that were observed after the treatment with SB-75 continuously released from the minipumps. This means that a constant fairly high serum level of this LH-RH antagonist is needed for an effective suppression of meiotic activity. Therapeutic serum levels of SB-75 can be even more conveniently and efficaciously achieved by the periodic (once a month) administration of microcapsules of SB-75. Advanced prototypes of microcapsules of SB-75 have been recently developed in collaboration with Cytotech and tested in our laboratory.

The agonist [d-Trp^6]LH-RH administered at the dose of 25 µg/day from the constant release microcapsules did not influence the spermatogenesis in our experiment. This confirms our previous finding that pretreatment with [d-Trp^6]LH-RH microcapsules had only a marginal protective effect against x-radiation-induced testicular damage in rats in contrast to the LH-RH antagonist (13). daCunha et al. (19) also failed to prevent testicular damage in mice caused by cyclophosphamide using the LH-RH agonist [d-Leu^6]LH-RH ethylamide. On the contrary, antagonistic analogs, like SB-75, appear to be able to suppress spermatogenesis, even when administered in mice in a continuous fashion. In view of the powerful testicular suppression achieved when the antagonist SB-75 was used in the form of a delayed delivery system, it is also clear that the failure of other investigators to obtain inhibition of the pituitary–gonadal axis in nude mice with an LH-RH antagonist was due to the fact that they used daily injections (20) and not continuous long-acting formulations.

The antifertility effect of various LH-RH antagonists with or without testosterone supplementation has been reported by our group and others (6–12) using rats and monkeys as test objects. Our present study confirms these observations and provides a less expensive and more flexible model—i.e., the mice—for the study of the activity and mode of action of LH-RH antagonists on germ cells. One of the possible uses of the mouse model could be for the investigation of the prevention of damage to germ cells during treatment with cytotoxic agents. Pretreatment with powerful antagonists like SB-75 for the prevention of gonadal damage inflicted by chemotherapy can also be studied in other models such as the rat (14) or the monkey (21). High activity of our antagonist SB-75 and related analogs (16) and the absence of side effects suggest a variety of practical clinical applications in the fields of oncology, gynecology, and reproduction (15, 22).

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