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Original Paper

Pharmacological Studies with Cetrorelix (SB-75), a Potent Antagonist of Luteinising Hormone-releasing Hormone

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The antitumour and hormone-suppressive effects of the luteinising hormone-releasing hormone LH-RH antagonist Cetrorelix (D-20761) and its pamoate salt (D-20762) were investigated in the model of the DMBA-induced mammary carcinoma of female rats and by testosterone determinations in normal male rats. Treatment with single high doses of D-20761 induced a rapid decrease of tumour weights with a dose-dependent duration of action. Strong antitumour effects were also observed by applying different multiple dose schedules, including a initial high dose (3.16 mg/kg, s.c.) followed by a low maintenance dose (31.6 μg/kg, s.c.). The stability of the molecule against degrading enzymes led to the idea of using the poorly soluble pamoate salt for facilitating a sustained release of active compound. This salt indeed induced a prolonged suppression of tumour growth and of testosterone levels. In conclusion, we found that Cetrorelix is a highly effective LH-RH antagonist which should be further developed for the treatment of hormone-dependent diseases. Copyright © 1996 Elsevier Science Ltd

Key words: LH-RH antagonist, hormone-suppression, DMBA mammary carcinoma, slow-release formulations

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INTRODUCTION

THE RELEASE of the gonadotropins luteinising hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary gland is regulated by a peptide hormone named LH-RH (LH-releasing hormone). In 1971, LH-RH was isolated from hypothalamic extracts and its amino acid sequence was established by Schally and associates [1]. Replacement of different amino acids then led to the discovery of highly active analogues like the superagonists, which have become indispensable in the clinical management of hormone-dependent diseases [2–4]. To achieve a suppression of the gonadotropins, a chronic administration of these compounds is necessary in order to induce a downregulation of pituitary receptors [5, 6]. The use of agonists initially leads to a stimulatory effect on LH, FSH and sex steroid secretion, which might result in a "flare up" of the disease [7]. The clinical advantage of antagonistic analogues is the immediate onset of gonadal steroid suppression after a single administration. There is evidence that LH-RH antagonists prevent LH-RH action on the pituitary by competitive binding to the LH-RH receptors. However, low potency of earlier antagonists, side-effects such as oedema due to histamine release and high dosage requirements have delayed the clinical development of LH-RH antagonists [8–10]. With the synthesis of compounds like Nal-Glu [11] or Antide [12] these side-effects started to decrease. Major improvement was achieved by the synthesis of the new LH-RH antagonist SB-75 (INN:Cetrorelix), by Bajusz and Schally [13].

As we have previously reported, Cetrorelix is free of anaphylactoid effects and produces strong suppression of gonadotropins [14, 15]. In addition, it is highly active in growth inhibition of the dimethylbenzanthracene (DMBA)-induced oestrogen-dependent mammary carcinoma of rats [16]. In this paper, further experimental results with Cetrorelix in the treatment of this particular tumour are described, using single high doses and different dose schedules. First experimental attempts towards the development of a depot-formulation are also reported.

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MATERIALS AND METHODS

Animals

Three- to four-month-old female Sprague–Dawley rats (Moellegaard Breeding Center, DK-8100 Ejby) weighing 250–290 g were used throughout the experiments. All animals were kept under specific pathogen free (SPF) conditions, fed with standard food pellets (Altromin 1324) and provided with drinking water *ad libitum*.

Compounds

The LH-RH antagonist Cetrorelix [Ac-D-Nal(2)1-D-Phe(4Cl)2-D-Pal(3)3-D-Cit6-D-Ala10]-LH-RH was used as its acetate salt (D-20761) and was synthesised by ASTA Medica AG, Germany. This salt has a good solubility and stock solutions of 10 mg/ml were prepared in 5.2% aqueous mannitol. Dilutions were made for each dose level to give a constant injection volume of 0.2 ml/rat. Cetrorelix pamoate (D-20762) has a poor solubility in water and was tested either as a suspension in tragacanth or formulated as particles of defined size (ASTA Medica AG).

[Ac-D-Nal-D-(pCl)Phr-D-Pal-Ser-Lys(Nic)-D-Lys(nic)-Leu-Lys(iPr)-Pro-DLAla]-LHRH (Antide) was purchased from Bissendorf Biochemicals GmbH, Hannover, Germany. The preparation of Antide was performed as described for Cetrorelix. All injections were prepared immediately before they were used.

Experimental procedure

The efficacy of Cetrorelix treatment was tested on autochthonous DMBA-induced mammary carcinoma in female Sprague–Dawley rats. The rats were given a single oral dose of 20 mg DMBA/animal at the age of 50 days. The first mammary tumours were detected 20–30 days later. The tumour weight was determined by palpation according to the method described previously [17], comparing the volume of each tumour to that of preformed plasticine models. The tumour weight was calculated by multiplication of the model weight by a factor which takes into account the specific weights of plasticine and tumour tissue. Validation of this method was performed by correlation of 99 palpated tumour weights with direct weighing of tumours after their excision. The correlation coefficient was 0.98.

After the total tumour mass per animal had reached about 1 g, the animals were randomly divided into treatment or control groups, consisting of seven rats each, and the treatment started on the same day. LH-RH analogues were injected subcutaneously (s.c.) daily for a time period as indicated in each experiment. All controls received injections of the vehicle alone. In some experiments, the number of single tumour-nodules was observed during the course of treatment.

Treatment with single high doses of Cetrorelix acetate (D-20761)

The efficacy of single high doses of Cetrorelix was assessed by s.c. injection of 1.0, 3.16 and 10 mg/kg. Additional groups were treated with the LH-RH antagonist Antide. Since the molecular weight of this antagonist is nearly identical to that of Cetrorelix, the same doses were used.

Efficacy of different dose schedules of Cetrorelix acetate (D-20761)

Different dose schedules of Cetrorelix were tested in the DMBA-tumour according to the method described above. During treatment with various dose schedules of D-20761, the antitumour effect and its duration was recorded. The treatment schedules tested consisted of: (1) a dose of 1 mg/kg s.c. given once a week for a total of 16 weeks; (2) starting with the single dose of 3.16 mg/kg on day 0 and continued with a dose of 316 μg/kg given twice a week from day 7 to 21; (3) starting with the single dose of 3.16 mg/kg on day 0 and continued with 31.6 μg/kg daily from day 7 to day 21.

Comparison of Cetrorelix acetate (D-20761) and Cetrorelix pamoate (D-20762)

Cetrorelix acetate and pamoate were compared for their antitumour efficacy and testosterone suppressive effects in rats. Five animals per group were injected with the different formulations s.c. into the right flank. For all manipulations, the animals were handled by two people to avoid major stress. The doses used were 0.5 and 1.5 mg/kg for testosterone measurement and 10 mg/kg for the assessment of the antitumour activity in the DMBA tumour (all single doses). Before administration to each animal, the pamoate solution was briefly stirred with a vortex and shaken gently several times before aspiration into the syringe. This optimally dispersed formulation was then applied to the animals according to the individual body weight. Blood samples were taken under anaesthesia from the sublingual vein at: 0, 6, 10, 24, 48, 72, 96, 120, 144 h etc. to a maximum of 21 samples in 21 days. The samples were collected in an Eppendorf tube, left at room temperature for 30 min and then centrifuged for 10 min at 3100 U/min. The serum was removed and centrifuged again for 3 min to obtain a clear sample. This was stored at −20°C for a maximum of 1 week before analysis of testosterone.

Measurement of testosterone by EIA

Testosterone was measured using an enzyme-linked immunoassay (EIA). This assay was provided by DRG Instruments GmbH. The test runs on microtitre 96-well plates. The amount of bound enzyme-conjugate is inversely proportional to the testosterone concentration of the sample. Absorption was measured at 450 nm. Evaluation of the microtitre plates was performed in a Mikrotrak EIA System (Syva) and evaluated via a software based on Excel. The specificity of the test is >98%. The standards used were: 0, 0.18, 0.50, 1.50, 5.00, 15.0 ng/ml. In every experiment, normal serum was used as an internal standard.

Measurement of Cetrorelix plasma level

Cetrorelix plasma levels were determined by using an radioimmunoassay originally developed by Schally and associates [18] and performed at the Institute of Physiology, University of Marburg.

In vitro *stability testing*

The stability of Cetrorelix acetate to exposure of proteolytic enzymes was also tested. The substance was disolved in 0.01 M phosphate buffer at a concentration of 1 mg/ml. This phosphate buffer was also used for adding the enzymes at a final concentration of 0.1 mg/ml. The pH values of the buffer solutions were adjusted to the corresponding optimum of each enzyme by the use of 5% phosphoric acid. The test-solutions were incubated at 37°C in a water bath. Samples were taken after 2, 6, 26 and 50 h, diluted 1:6.6 with 30% acetic acid and analysed immediately by high pressure liquid chromatography for the determination of content expressed in percentage of initial amounts. The enzymes employed were: chymotrypsin from bovine pancreas (Boehringer Mannheim), pH optimum at 8.0; Pronase from *Streptomyces griseus* (Boehringer Mannheim), pH optimum at 7.0; Subtilisin from *Bacillus subtilis* (Boehringer Mannheim), pH optimum at 9.0. For comparison, the commercially available LH-RH agonist Buserelin acetate (Suprefact ®), a nonapeptide with one D-aminoacid (Glp-His-Trp-Ser-Tyr-D-Ser(-*o*-but)-Leu-Arg-Pro-NH-C_2H_5), was tested according to the same procedure.

RESULTS

Treatment with single high doses of Cetrorelix acetate (D-20761)

When single high doses of 1.0, 3.16 and 10.0 mg/kg were injected s.c., a dose-dependent duration of growth inhibition and tumour regression was found (Figure 1a). With the dose of 3.16 mg/kg, the tumours regressed starting on day 3 after treatment and tumour growth remained suppressed for an additional 14 days. When the higher dose of 10 mg/kg was used, these effects were even more pronounced and produced tumour inhibition for 43 days. Only after 60 days did the tumour weights increase to levels corresponding to those before treatment.

The LH-RH antagonist Antide, which was tested in comparison, was less effective with regard to both parameters, tumour regression and duration of tumour growth inhibition (Figure 1b). The dose of 1.0 mg/kg was ineffective, whereas a dose of 3.16 mg/kg showed relatively minor antitumour response. The dose of 10 mg/kg lead to tumour regression, which lasted 20 days before regrowth occurred.

Efficacy of different dose schedules of Cetrorelix acetate (D-20761)

Figures 2 and 3 show the tumour growth curves after treatment with different dosages of Cetrorelix. DMBA-induced mammary tumours can very effectively be treated with the various dose schedules. Tumour growth inhibition and tumour regressions were obtained in all experiments.

The antitumour response obtained with a single dose of 1 mg/kg could be maintained and even became more pronounced when this dose was given once a week for 4 weeks (Figure 2). The tumours started to regress after day 4 and reached the limit of detection (<0.1 g) within 14 days. One week after termination of treatment, the tumours started to regrow.

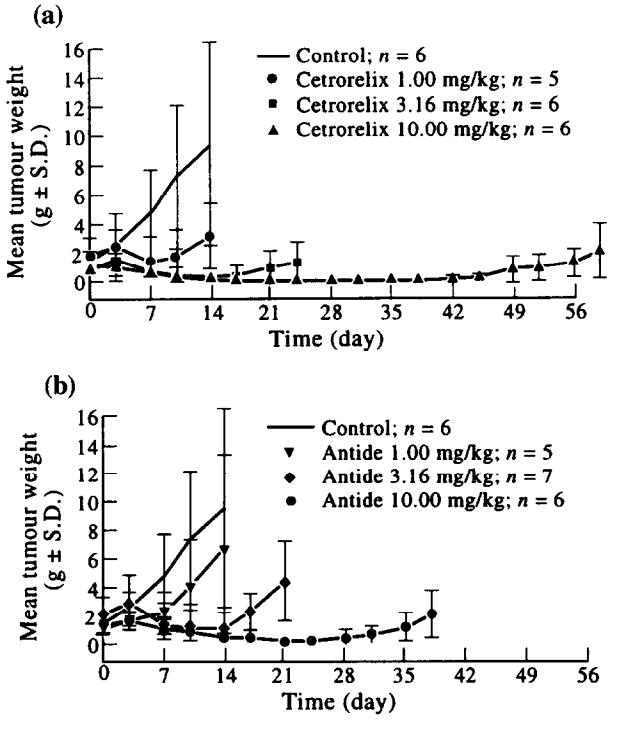

Figure 1. (a) Efficacy of single high doses of Cetrorelix (D-20761) s.c. in the DMBA mammary tumour model. (b) Efficacy of single high doses of Antide s.c. in the DMBA mammary tumour model.

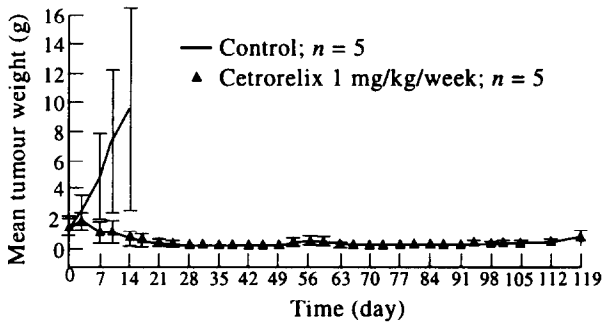

Figure 2. Antitumour effects of a weekly treatment with 1 mg/kg s.c. Cetrorelix (D-20761) in rats bearing DMBA-induced mammary tumours.

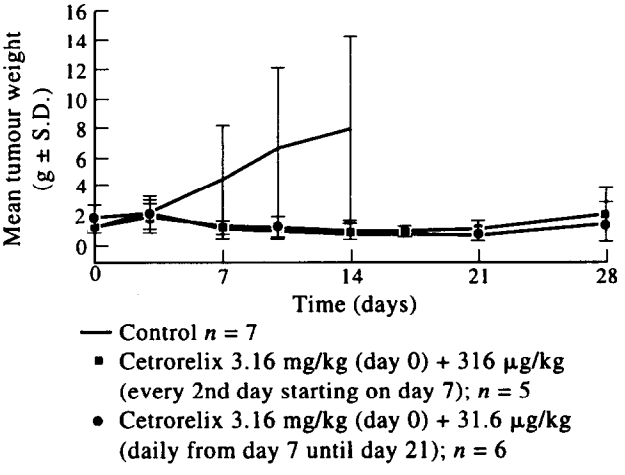

Figure 3. Antitumour efficacy of a loading dose/maintenance dose schedule in the DMBA mammary tumour model using a loading dose of 3.16 mg/kg s.c. (day 0) and a maintenance dose of either 31.6 µg/kg s.c. per day or 316 µg/kg s.c. given every second day starting on day 7 (D-20761).

We then examined the efficacy of dose fractionation. When the treatment was started with a single loading dose of 3.16 mg/kg, surprisingly, a daily dose of 31.6 µg/kg, equivalent to 1/100 of the initial dose and given from day 7 to 21, was effective in maintaining tumour regression (Figure 3). Although treatment with the maintenance dose was terminated on day 21, tumour growth was inhibited for an additional 7 days, before regrowth started. By using the dose of 316 µg/kg twice a week, after initial 3.16 mg/kg, a slightly less pronounced efficacy was observed, with tumour weights declining to 0.5 g (Figure 3). We also observed that the change in net body weight during treatment reflected the activity of the antagonists: net body weight increased parallel to tumour regression and tended to decrease after regrowth of the tumours.

In vitro *stability of Cetrorelix to proteolytic enzymes*

The results of the peptide content determinations by HPLC showed that during a period of 50 h at 37°C, the content of Cetrorelix remained virtually stable after incubation with chymotrypsin, pronase and subtilisin (data not shown). Degradation products were found only in the range 0.8–1.5%. In contrast, the LH-RH agonist Buserelin was almost

completely degradated by all three enzymes within 2.5 h of incubation. When both compounds were tested in the presence of human serum, Cetrorelix again proved to be stable over the test-period of 50 h, whereas Buserelin was decomposed by 30%.

Antitumour response of Cetrorelix pamoate (D-20762)

Pamoate compared to acetate. Both salts of Cetrorelix were tested using equimolar doses with regard to the peptide-base. DMBA mammary tumours were effectively treated with D-20761 or D-20762 at a dose of 10 mg/kg (data not shown). The duration of the tumour growth inhibition was longer for D-20762 as compared to D-20761, with regrowth of the tumours to baseline after 70 days for the pamoate versus 42 days for the acetate (data not shown). In terms of reduction in tumour weight, the efficacy of D-20762 treatment was superior to that with D-20761. At the nadir of tumour growth inhibition (day 28), an average number of only 0.57 tumour-nodules were counted in animals of the group treated with D-20762, whereas it was 2.14 in the group treated with D-20761.

Pamoate in defined particle size. As described above, a prolonged activity of Cetrorelix was observed using the pamoate salt. However, efforts towards producing a more uniform material led to formulations with a defined particle size. The size which produced the most effective and long lasting testosterone suppression was up to 125 μm. At a dose of 0.5 mg/kg s.c., testosterone was suppressed in all animals, but in 3/5 animals the suppression lasted 360 h and the hormone values increased thereafter (Figure 4b). In two animals only a short term suppression was observed, resulting in lower but not complete suppression of testosterone. Determination of plasma levels of Cetrorelix indicated that the observed lack of activity in these animals was not due to an inadequate dosage or that the compound was not systemically available. We assumed that 0.5 mg/kg represents a lower-limit dose, since the use of 1.5 mg/kg produced uniform testosterone suppression. With the use of this dosage, a suppression of testosterone down to the limit of detection (0.2 ng/ml) was achieved for a period of at least 31 days (Figure 4b).

Plasma level determinations from the treatment with 1.5 mg/kg (Figure 5a) revealed that the acetate salt of Cetrorelix produced very high initial concentrations of about 100 ng/ml 24 h after injection, with a rapid decline to about 3 ng/ml at 144 h, and it can be assumed that the limit of detection (0.28 ng/ml) was reached at 192 h. The slow release formulation of the pamoate salt showed a low initial liberation corresponding to about 15 ng/ml and a subsequent continuous release, with Cetrorelix levels declining to about 3 ng/ml at 336 h and reaching the limit of detection at 576 h.

Twenty-four hours after injection, comparable plasma levels were found for the group treated with 0.5 mg/kg of the pamoate, but levels were lower at later time points and about 3 ng/ml were reached after 192 h (Figure 5b).

DISCUSSION

A strong inhibition of LH levels has been observed by Cetrorelix acetate administration in several animal models and humans [19, 20]. It was previously found that a daily s.c. injection of 100 μg/kg of Cetrorelix, was the minimum amount capable of exerting a full antitumour response in the DMBA-induced mammary carcinoma, and that these antitumour effects were accompanied by the appearance of marked apoptosis [16]. Similar observations were made in other transplantable hormone dependent tumour models, in which Cetrorelix was effective [21, 22].

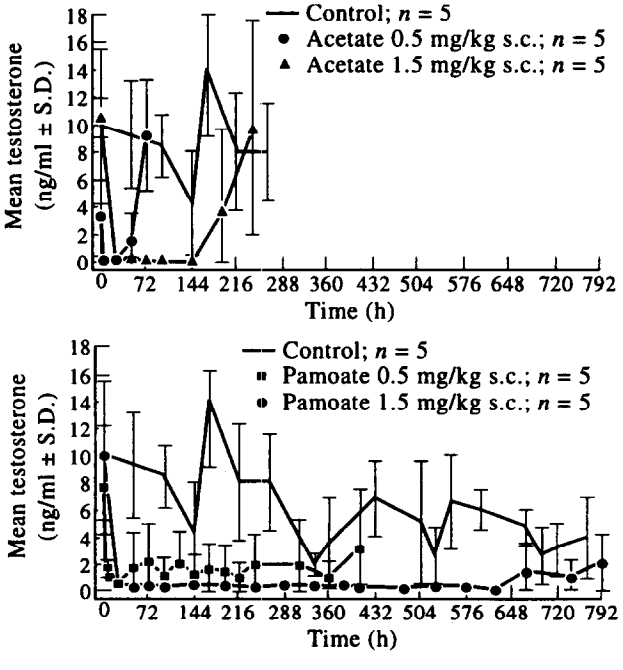

Figure 4. (a) Testosterone levels in normal male rats after single s.c. doses of 0.5 or 1.5 mg/kg Cetrorelix acetate (D-20761). (b) Testosterone levels in normal male rats after single s.c. doses of 0.5 or 1.5 mg/kg Cetrorelix pamoate (D-20762).

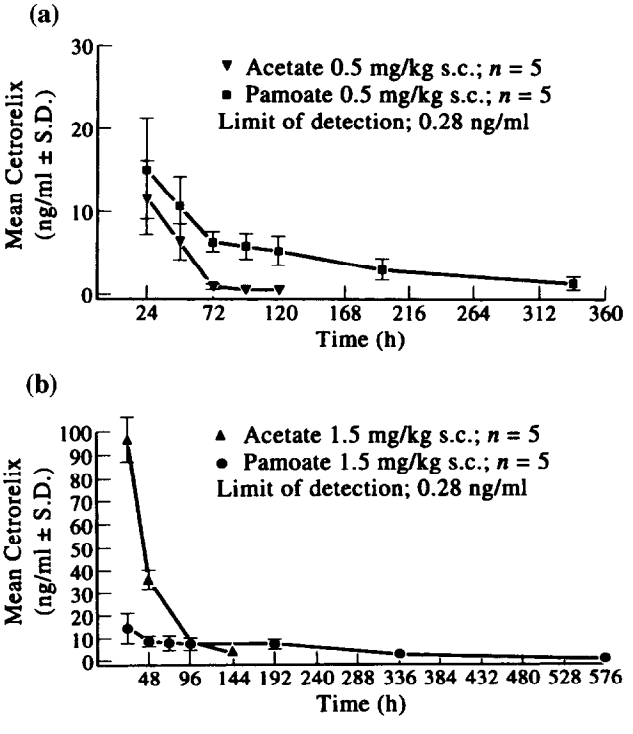

Figure 5. (a) Plasma levels of Cetrorelix after single s.c. administration of Cetrorelix acetate (D-20761) or Cetrorelix pamoate (D-20762) at a dose of 0.5 mg/kg starting 24 h after injection. (b) Plasma levels of Cetrorelix after single s.c. administration of Cetrorelix acetate (D-20761) or Cetrorelix pamoate (D-20762) at a dose of 1.5 mg/kg starting 24 h after injection.

By using the acetate salt of Cetrorelix, we have previously demonstrated that with the daily 100 μg/kg dose, even a very large tumour burden of 6 g can effectively be treated. During daily therapy for 5 weeks, no escape from tumour growth inhibition was observed [16]. A comparable result was obtained with the weekly s.c. injection of 1 mg/kg over 6 weeks. The injection of single high doses was also very effective in the induction of tumour regression. In comparison, the LH-RH antagonist Antide was less effective with regard to antitumour efficacy and duration of action. Similar results were reported from studies in other models [23, 24]. The comparatively long efficacy of Cetrorelix acetate might be explained by the presence of five D-amino-acids within the molecule, resulting in an extended half-life. In addition, the formation of a subcutaneous depot cannot be excluded. In first clinical trials, an elimination half-life of 30 h was found in healthy male volunteers after a single s.c. injection of 5 mg [25].

Based on the finding of this extended half-life in humans, we evaluated in rats the antitumour effects of a dose schedule consisting of a high loading dose followed by a low maintenance dose, in which the latter was not active by itself. The efficacy of such a regimen was indeed demonstrated. Although the treatment with the maintenance dose was terminated on day 21, the tumours were inhibited for an additional 7 days before they started to regrow. These results cannot be interpreted only in terms of competitive receptor blockade. Thus, there are three possible mechanisms which can explain the observed effects singly or in combination.

Firstly, there may be downregulation of LH-RH receptors, which takes place after the initial high dose treatment. Evidence for such an effect was recently provided by Srkalovic and associates [22]. Subsequently, a low dose which is not active by itself, is sufficient to maintain this status and a consequent tumour growth inhibition.

Secondly, a comparison of the acetate salt and the pamoate formulation of Cetrorelix indicates that pharmacokinetic effects might well contribute to the observed effects. In the experiments described, we found that high plasma levels at the start of the treatment and a rapid decrease thereafter, as produced by Cetrorelix acetate, were not sufficient to maintain a continuous suppression of testosterone. Therefore, the time of exposure to a certain plasma level of antagonist at the beginning of the treatment is of importance. This possibly induces a mechanism, including a downregulation of LHRH receptors, allowing the effectiveness of subsequent very low doses. Since the effective plasma levels measured for the depot formulation were low as compared to those of aqueous solution of the acetate salt, an accumulation of Cetrorelix might occur within a pituitary compartment.

Finally, a dose- and time-dependent inhibition of gonadotropin-subunit synthesis in the gonadotrophic cells of the pituitary may be involved. Subsequently, this inhibition can be maintained by low doses of the antagonist.

The results described clearly show that the pamoate salt of Cetrorelix has the effect of a slow release formulation. The range of variation in the hormone suppression of individual animals could be reduced by using the pamoate salt of Cetrorelix at a defined particle size of up to 125 μm. Plasma level measurements of this formulation revealed a small initial liberation of Cetrorelix and a subsequent long period of release. According to the results described for the dose schedules of the acetate salt, this initial higher amount of substance released is needed in order to maintain the pharmacodynamic effects with much lower plasma levels thereafter. The variations in the duration of testosterone suppression in the 0.5 mg/kg group can be explained by regarding this dose level as borderline in efficacy, since further increase in dosage led to more homogeneous results with respect to testosterone suppression.

In all experiments, the treatment with Cetrorelix was well tolerated and no obvious side-effects, even with high doses, were observed. The presented data for Cetrorelix show its high effectiveness in producing suppression of gonadotropins and sex steroids which can induce a marked antitumour response. From clinical data of phase I and phase II studies, it appears that Cetrorelix is safe and effective and should be useful in the treatment of hormone-dependent malignant and benign diseases [25, 26].

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