Systemic Delivery of Cetrorelix to Rats by a New Aerosol Delivery System

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Purpose. To study the pulmonary absorption and tolerability of various formulations of the decapeptide cetrorelix acetate in rats by a new aerosol delivery system (ASTA-ADS) for intratracheal application.

Methods. Using the ASTA-ADS, cetrorelix liquid formulations (aqueous solutions for ultrasonic nebulization) were firstly selected and subsequently delivered as nebulized aerosol to orotracheally cannulated rats. The pharmacologic effect (decrease of testosterone serum level) of four cetrorelix formulations was determined in rats by enzyme linked immunosorbant assay, and pharmacokinetic data were determined after measurement of cetrorelix serum level by radioimmunoassay. Histological examination of the lung was performed at the end of the experiments, and in a supplementary experiment the respiratory parameters (resistance and compliance) of rats were monitored by a validated pulmonary monitoring system during the aerosol application of the same formulations.

Results. After an exposure time of 5 min, the applied formulations reduced the testosterone concentration in serum to subnormal levels (≤1 ng/ml) over a period of 24 h. Comparing the plasma concentration after intratracheal aerosolization with data of intravenous administration, the mean calculated bioavailabilities for the four formulations using the corrected dose (delivered—exhaled amount) were between 48.4 ± 27.0% and 77.4 ± 44.0%. The histologic examination of the lungs revealed different tolerability of the various tested formulations ranging from locally intolerable to well tolerated. The measurement of the lung function parameters did not reveal any compound or formulation related changes.

Conclusions. Our studies show that cetrorelix can be effectively administered as aerosol and that intratracheal aerosolization via the ASTA-ADS provides results that are well comparable to other application routes, as demonstrated by statistical comparison of the newly obtained data with previous results from intratracheal instillation of cetrorelix solutions in rats.

KEY WORDS: GnRH-analogues; cetrorelix; inhalation aerosol; pulmonary peptide delivery.

INTRODUCTION

Over the last decade, growing attention has been given to the potential of the pulmonary route as an alternative noninvasive means for systemic delivery of macromolecules, especially of peptide and protein-based therapeutic agents (1–4).

Preclinical studies in animals frequently use intratracheal (i.t.) instillation or aerosol delivery to determine pulmonary absorption and bioavailability (5–8). Intratracheal instillation is not a physiologic route of application, and the results obtained may not be transferable to aerosol application in humans, because of discrepancies in particle distribution, clearance, the possibility of injury, and varying bioavailability between instillation and aerosol delivery (9,10). In opposition, by aerosol delivery, the delivered amount of drug does not necessarily correspond to the deposited dose at the desired administration site because of substance loss in the delivery system, in the oropharyngeal region, and due to exhalation of a part of the delivered aerosol (11,12). In our laboratories, we have developed a new system of endotracheal aerosol delivery (13), which allows the exact calculation of both, the amount of drug delivered to the lung of laboratory rats, and the exhaled amount. Therefore, it allows the calculation of the amount of drug effectively deposited in the lung. Using this system, we examined the absorption of four aerosol formulations of the decapeptide cetrorelix acetate (referred to as cetrorelix) in anesthetized rats.

Cetrorelix, a potent antagonist of the gonadotropin-releasing hormone (GnRH) (14), seems to be suitable for therapy of sex-hormone dependent pathologies, such as prostate carcinoma and treatment of infertility (15). The systemic absorption of cetrorelix via the lung has previously been demonstrated in sheep (16) and in rats (8) by i.t. instillation. In our study, both cetrorelix and testosterone serum concentrations were determined after i.t. aerosol administration to rats. We were able to show that after an application time of 5 min, cetrorelix was absorbed from the lungs at pharmacologically active concentrations, leading to depression of testosterone plasma concentrations to subnormal levels (≅1 ng/ml). Additionally, pharmacokinetic analysis and histological examination were performed. In a supplementary series of experiments, the same cetrorelix formulations were tested in rats by a validated pulmonary monitoring system to determine the influence of their aerosolization on lung function parameters.

MATERIALS AND METHODS

Materials

The peptide cetrorelix was manufactured by ASTA Medica AG (Frankfurt Main, Germany) in the form of a lyophilisate to be reconstituted with water or an appropriate medium (Table I). The solutions for the nebulization in the *in vivo* experiment were obtained by reconstitution of previously prepared cetrorelix lyophilisate (as vials containing 22.5 mg of cetrorelix, calculated as cetrorelix base) with 15 ml of the respective medium immediately before the application to obtain a solution of 1.5 mg/ml cetrorelix. Atropin, used for the premedication, was purchased from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). Xylazine (Rompun®) and Ketamine (Ketavet®), used for the anesthesia, were purchased from Bayer AG (Leverkusen, Germany) and Upjohn GmbH (Heppenheim, Germany), respectively. The oral endotracheal intubation was performed using a vein catheter of 50 mm length, 1.7 mm i.d. and 2.1 mm o.d., from B.

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Table I. Cetrorelix Formulations as Aqueous Solutions (1.5 mg/ml) with Adjuvants for Ultrasonic Nebulization, and Corresponding Aerosol Characteristics

	Composition			Aerosol characteristics		
	Ethanol (V/V %)	Tween-80 (W/V %)	α-Lecithin (W/V %)	Output[a] (mg)	MMAD[b] (μm)	GSD[b]
Form. 1	–	0.05	–	5.32	4.0	1.9
Form. 2	10	0.05	–	5.67	5.0	2.0
Form. 3.	10	–	0.01	4.16	4.9	2.0
Form. 4	10	0.05	0.01	6.09	4.9	2.0

[a] 10 min nebulization at 1.68 l/min (24 ml/stroke at 70 strokes/min).
[b] 10 min nebulization at 30 l/min.

Braun Melsungen AG (Melsungen, Germany). The determination of testosterone serum levels was carried out by an enzyme-linked immunosorbant assay (ELISA); the kit used (Testosteron EIA) was purchased from DRG Instruments GmbH (Marburg, Germany); samples were measured using the ELISA workstation from SYVA Diagnostica (Darmstadt, Germany). The other reagents and substances used for the preparation of the various formulations, as well as the solvents used for the high performance liquid chromatography (HPLC) were purchased from Merck AG (Darmstadt, Germany). Immunoreagents, radioligands, and other components for the radioimmunoassay (RIA) of cetrorelix were supplied by ASTA Medica AG. The radioactivity of the samples was measured by a Gamma Counter Wallac 1470 Wizard (Berthold, Wildbad, Germany). Urethane and gallamine used for the anesthesia in the lung function experiments were purchased from Sigma-Aldrich (Deisenhofen, Germany). The measurements of lung function parameters were performed by a validated pulmonary monitoring system (PMS) established at AWD GmbH, Radebeul, Germany.

Animals and Husbandry

Male Sprague Dawley rats weighing between 300 and 370 g were purchased from Mollegaard-Bomholtgaard (Ry, Denmark), maintained under specific pathogen-free (SPF) conditions and used for experiments after an acclimatization period of about 1 week after arrival.

Animal experiments were performed according to the "Principles of Laboratory Animal Care" enforced by German Federal Regulations. The experimental protocol for pharmacologic and pharmacokinetic studies has been approved by the Animal Protection Committee of the State of Hessen, Germany and the protocol for lung function has been approved by the authorities of the state of Saxony, Germany.

Apparatus Description

The ASTA-ADS consists of a rodent ventilator, which delivers a fixed volume of air in a pulsating manner to an ultrasonic nebulizer, which in turn generates the aerosol (Fig. 1). The flow of aerosol reaches a flow splitter where it is split up in four equal parts directed to a cubical plexiglas box containing three anesthetized and orotracheally cannulated rats. The animals receive the aerosol directly from the splitter using Tygon pipes and Y pieces connected to an intratracheal cannula.

The box also contains two wash-bottles of 500 ml each. One bottle (referred to as WB_{inh}) receives a quarter of the total delivered aerosol directly from the splitter and permits the determination of the delivered amount of substance by measurement of its concentration in the washing fluid. The other bottle (referred to as WB_{exh}) receives the exhaled aerosol from the rats via the third arm of the Y piece. The amount of exhaled agent allows calculation of the mean deposited dose of aerosol into the rat lungs (see formula in Fig. 1). The exits of both wash-bottles are connected to a drying tube and the dried, filtered air is transported to the pump.

Formulations Screening

Various cetrorelix solution for ultrasonic nebulization were tested by the ASTA-ADS (Fig. 1) *in vitro* for determining output. Unfortunately, not all cetrorelix solutions can be efficiently nebulized, because of the characteristic of cetrorelix to aggregate in water and to generate gel (8). The gel formation, in turn, increases the viscosity and could be responsible for the ineffectiveness of the ultrasonic nebulization (17,18). Solutions of cetrorelix in water with a concentration higher than 1 mg/ml were incapable of generating an adequate amount of aerosol, and various coadjuvants were tested to optimize its output. The nature and the concentration of the selected adjuvants for cetrorelix formulations, such as ethanol, Tween-80 and α-lecithin were selected from the literature (17,18,19) and by previous studies performed in our laboratory (data not shown). The various formulations were

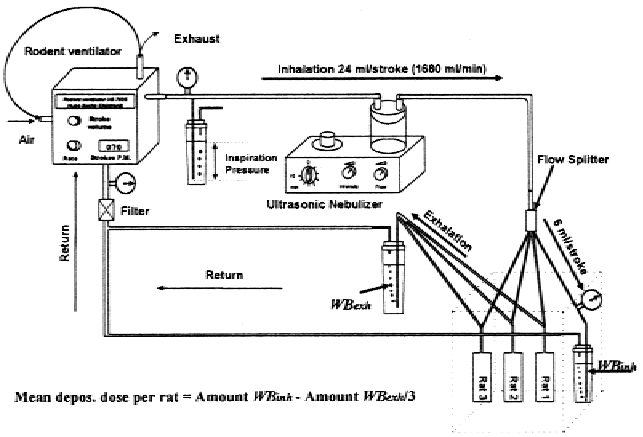

Fig. 1. Scheme of the ASTA Medica aerosol delivery system. For a better explanation of the device, the scale and the precise location of the various components are not respected.

nebulized for a period of 10 min in a pulsatile manner using a frequency and a stroke volume identical as those used for the *in vivo* application by the ASTA-ADS. The cetrorelix concentration in the washing fluid of both wash-bottles was determined by HPLC. The separation was performed using a prepackaged column type "EP 125/4 Nucleosil 120-3 C18" (Macherey-Nagel GmbH, Düren, Germany) and a mobile phase consisting of H_2O/CH_3CN (365/635 ml) and 1 ml CF_3COOH. The column was maintained at 40 °C, and the detection performed at 226 nm. In parallel, the HPLC analysis allowed determination of the stability (expressed as purity) of cetrorelix after nebulization by comparison to the non-nebulized standard. The composition and the total output of the formulations selected by the previously mentioned screening are shown in Table I. The placebo solutions used for the control groups consisted of isotonic saline, isotonic mannitol solution, and the reconstitution medium of form. 4 (Table I).

Moreover, the aerosols of these cetrorelix formulations were characterized by a four-stage liquid impinger (Fisons Scientific Apparatus, Leicestershire, UK) in which a supplementary glass-fiber filter (Type GF/, Whatman, UK) was coupled to the terminal end of the impinger as previously described by Evans *et al.* (20). When operated at 60 l/min airflow by a vacuum pump downstream of the filter, the effective cutoff diameters (ECD) for stages 1–4 were 10.47, 5.51, 3.59, and 1.25 μm, respectively (20). Each stage was filled with 10 ml 15% (V/V) acetic acid, and the ultrasonic nebulizer was connected via a "glass throat" to the impinger. A flow of 30 l/min was used, and a correction of the cutoff was performed using the formula:

$$ECD_{30\ l/min} = ECD_{60\ l/min} * (60/30)^{1/2} \quad (1)$$

as prescribed by the European Pharmacopoeia. The data were expressed as percentage of the deposition on the four stages and on the filter of the liquid impinger. The mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD) of the aerosol droplets were estimated by construction of particle size-cumulative undersize plots using a log probability graph.

In Vivo Experiments

Eight groups of rats were used for the *in vivo* experiments. Four groups ($n = 9$, each) were used for the aerosol application of the four cetrorelix formulations, and three groups ($n = 6$, each) were used for the application of the three different placebo formulations as control for the aerosol application (in the following referred to as placebo groups). An additional group of rats ($n = 5$) served as an untreated control group (in the following referred to as control group) for the histological examination. In this latter group, the animals were only anesthetized and did not receive any aerosol.

At the start of each experiment, the animals were weighed and medicated with Atropin by i.m. injection of 50 mg/kg body weight to facilitate the orotracheal intubation and the subsequent aerosol application (13,21).

After 10 min, they were anesthetized with a mixed solution of xylazine (8 mg/kg body weight) and ketamine (80 mg/kg body weight) by i.m. injection. An orotracheal catheter was inserted as described in a previous work (21). The animals were then kept in a heated box (37°C) until the treatment.

The treatments with the ASTA-ADS were performed as follows: approximately 250 ml of acetic acid solution were placed in each of the two wash-bottles, and approximately 15 ml of the test solution were filled into the chamber of the atomizer as described elsewhere (13).

The ventilator was set at a volume of 24 ml per stroke (6 ml for each inhalation line) at a frequency of 70 strokes per minute. After starting the ventilator, the rats were placed on the plexiglas box and connected to the three Y joints of the system.

One minute after the start of the mechanical ventilation, the atomizer was activated and the aerosol was applied for 5 min. The rats were then disconnected from the apparatus and returned to the heated box for recovery. The other rats with the exception of the control group were treated similarly. The animals were then subjected to monitoring of testosterone concentrations by blood sampling from a sublingual vein under a light CO_2 narcosis, as also recommended by Kohler *et al.* (22) and Zeller *et al.* (23). Sampling times in all experiments were $t = 0$, 0.5, 1, 2, 6, 10, 24, 48, and 72 h. They were based on published methods (8,13,23), and restricted to less than seven blood samples per week. Consequently, the samples at $t = 0$ h were taken 1 week before the application, and the samples at $t = 72$ h were taken postmortem.

The blood sample volume was ≤500 μl each. After 10 min, each sample was centrifuged for 20 min at 4500 rpm, the pellet removed, and the supernatant centrifuged again for 3 min. The clear and colorless serum was split in two parts and then stored at −20°C until analytical determination. One fraction served for the determination of testosterone concentration by ELISA as pharmacologic parameter (Table II), the other one for the determination of cetrorelix concentration by RIA for the calculation of pharmacokinetics (Table III). This was performed using a validated Excel-application for non-compartmental analysis (Funcalc 1.01, calculation ASTA Medica AG, Germany).

The area under the serum concentration-time curve (AUC) and area under the first moment curve (AUMC) were calculated using the trapezoidal rule. Following i.t. aerosol administration, mean residence time (MRT) was calculated as AUMC/AUC; bioavailability was calculated as $[AUC_{i.t.a.} \times dose_{i.v.}]/[AUC_{i.v.} \times dose_{i.t.a.}] \times 100$ and the $t_{1/2}$ was calculated as $\ln 2/k$. The k was obtained from the regression curve calculated by the fitting of the last three points in the graphic concentration vs. time. The pharmacokinetic parameters of the i.v. and i.t. application used as comparison are obtained in a previous study (8) and data are reported in Table III.

One-way analysis of variance (ANOVA) and Dunnet's test were performed to demonstrate statistical differences using the software Sigma-Stat for Windows, ver. 2.03 (SPSS Inc., San Rafael, CA, USA).

Histological Examination

All five rats used as untreated control subjects for the histological examination as well as the first six animals of the other groups (placebo and verum groups) were sacrificed by CO_2 euthanasia 72 h after the treatment, and the trachea with lungs were removed. The lungs were gently inflated with 5–6

Table II. Testosterone Serum Concentration After 5 min Nebulization of Placebo and Cetrorelix Solution in Correlation with Testosterone Value After Intratracheal Instillation of 0.5 mg/kg Cetrorelix in Rats

Time H	Placebo[a] mean (SD)	I.t. inst.[b] mean (SD)	Form. 1 mean (SD)	Form. 2 mean (SD)	Form. 3 mean (SD)	Form. 4 mean (SD)	Statistical analysis ANOVA Cont. vs. verum	Statistical analysis ANOVA i.t. inst. vs. i.t.a.
0	3.4 (1.5)	12.4 (5.4)	5.1 (2.4)	2.4 (1.0)	4.6 (3.9)	2.3 (1.5)	No[c] $P > 0.05$	Yes $P < 0.001$
0.5	4.3 (1.9)	4.6 (3.1)	2.7 (1.0)	4.0 (2.9)	3.9 (2.8)	3.4 (2.4)	No $P = 0.429$	No $P = 0.657$
1	5.7 (0.8)	3.7 (2.9)	4.0 (1.8)	5.4 (2.8)	6.1 (4.0)	4.9 (3.4)	No $P = 0.539$	No $P = 0.488$
2	5.7 (1.3)	2.1 (1.6)	3.7 (1.9)	4.2 (2.1)	5.2 (3.5)	5.2 (3.4)	No $P = 0.386$	No $P = 0.158$
6	2.7 (1.9)	1.0 (0.4)	1.6[d,e] (0.3)	0.9 (0.2)	1.2 (0.6)	1.2 (0.5)	Yes $P < 0.001$	No $P > 0.05$
10	2.4 (1.5)	0.8 (0.2)	1.1 (0.3)	0.9 (0.2)	0.9 (0.2)	0.9 (0.2)	Yes $P < 0.001$	No $P = 0.092$
24	2.6 (1.5)	1.1 (1.3)	1.0 (0.5)	0.9 (0.5)	0.9 (0.3)	1.0 (0.4)	Yes $P = 0.001$	No $P = 0.614$
48	4.1 (2.2)	6.4 (1.1)	3.2[d] (2.6)	1.8 (1.2)	1.3 (0.8)	1.7 (0.8)	Yes $P = 0.002$	Yes $P < 0.001$
72	4.3 (1.2)		3.0 (1.8)	1.7 (0.5)	1.6[f] (2.3)	2.0 (1.1)	No $P > 0.05$	—

[a] Mean of three control groups (isotonic mannitol-, isotonic NaCl-, and hypotonic tween/lecithin-solution), six rats per group.
[b] Intratracheal instillation of 0.5 mg/kg cetrorelix as isotonic mannitol solution (data from Lizio *et al.,* 14).
[c] Multiple comparisons vs. control group (Dunnet's method).
[d] No statistical difference to the control group, $P > 0.05$ (Dunnet's method).
[e] Statistical difference to the i.t. instillation group, $P < 0.05$ (Dunnet's method).
[f] Statistical difference to the control group, $P < 0.05$ (Dunnet's method).

ml of 10% neutral buffered formalin (NBF) via intratracheal instillation and then immersed in 10% NBF until the histological examination. Tissues were processed routinely by paraffin embedding, sectioning at nominally 2 μm and staining with hematoxylin and eosin. Stained sections were examined for evidence of treatment-related morphologic effects by a German board-certified veterinary pathologist using light microscopy.

Table III. Group Distribution, Dosing Regimens, Statistic, and Pharmacokinetic Parameters as Mean Value ± Standard Deviation (SD) of Cetrorelix in Male Rats Following Intravenous Application (i.v.), Intratracheal Instillation (i.t. inst.), and Aerosol Delivery of Four Formulations (Form. 1–4)

Parameter	i.v.[a] mean (SD)	I.t. inst.[a] mean (SD)	Form. 1 mean (SD)	Form. 2 mean (SD)	Form. 3 mean (SD)	Form. 4 mean (SD)	Difference ANOVA i.t. inst. vs. i.t. aer.
Deliv. dose (mg/kg)	0.1	0.5	1.5 (0.2)	1.4 (0.03)	1.4 (0.1)	1.4 (0.1)	n.d.
Corr. dose (mg/kg)	0.1	0.5	0.6[b] (0.1)	0.6 (0.1)	0.5 (0.1)	0.5 (0.1)	No $P > 0.05$
No. of animals	4	7	9	9	9	9	—
C_{max} (ng/ml)	188.0 (67.3)	231.2 (47.0)	392.1 (354.8)	232.4 (154.4)	200.6 (171.5)	163.1 (95.3)	No $P = 0.256$
t_{max} (h)	—	0.8 (0.5)	1.3 (0.7)	1.3 (1.8)	0.9 (0.5)	1.0 (0.0)	No $P = 0.156$
$t_{1/2}$ (h)	2.1 (1.1)	10.8 (1.2)	20.9 (4.6)	17.9 (5.6)	22.9 (8.0)	22.8 (4.2)	Yes $P < 0.001$
$AUC_{0\to\infty}$ (ng × h/ml)	256.3 (61.3)	962.8 (176.9)	1252.8 (781.4)	906.5 (450.5)	571.5 (342.0)	648.3 (351.6)	No[c] $P > 0.05$
MRT (h)	1.9 (1.4)	5.4 (0.4)	7.4 (3.9)	12.4[b] (3.4)	9.9 (3.6)	13.3[b] (6.1)	No[c] $P > 0.05$
Abs. bioav. del. (%)	100	75.1 (13.8)	32.0 (19.0)	24.7 (12.5)	16.4 (9.2)	18.7 (10.5)	n.d.
Abs. bioav. cor. (%)	100	75.1 (13.8)	77.4 (44.0)	61.9 (27.0)	48.4 (27.0)	50.4 (29.5)	No $P = 0.207$

[a] Data from Lizio *et al.* (14).
[b] Statistically different, $P < 0.05$ (Dunnett's method).
[c] Multiple comparisons vs. i.t. inst. group (Dunnett's method).

Lung Function Measurement

Male Sprague Dawley rats were anesthetized with 1.5 g/kg urethane intraperitoneally. The left carotid artery was prepared and a catheter for continuous blood pressure measurement was inserted. The trachea was exposed during the preparation of the artery, cannulated, and connected to a small animal respirator (KTR 5, FMI, Engelsbach, Germany). To exclude spontaneous breaths, animals were paralyzed with 10 mg/kg gallamine intravenously. The animals were then artificially ventilated with a mixture of oxygen and ambient air (20:80) using a tidal volume of 6 ml at a frequency of 70 strokes per minute. After an equilibration period of about 10 min, the control solution (isotonic saline) was administered by inhalation using a computer controlled ultrasonic nebulizer, which was triggered by the inspiration flow (nebulizer on for 0.25 s after start of inspiration) for a time of 5 min (5 times 1 min of application with an interval of 30 s between the nebulizations). The animals were automatically ventilated for another 15 min (recovery time) and subsequently, in the same manner, the four cetrorelix formulations were applied. The respiratory flow (ml/s) was measured using a Fleisch-tube (Hans Rudolph Inc., Kansas City, KS, USA) in line with the respirator. The transpulmonary pressure (TTP = inflation pressure—intrathoracic pressure, cm H_2O) was measured continuously via side connection of the tube (inflation pressure) and an intrathoracic cannula (intrathoracic pressure) by a pressure transducer. From these data (tidal volume, flow, and TTP), lung resistance (R_L, cm H_2O/l/s) and dynamic lung compliance (C_{dyn}, ml/cm H_2O) were calculated for each respiratory cycle and recorded using a computer controlled system (PMS PR 800, Mumed System Ltd., London, UK).

Changes of R_L and C_{dyn} were calculated from the difference between baseline (20 breaths averaged before nebulization) and maximum effect within 5 and 10 min after nebulization (ten breaths averaged) and reported as percentage of rise of resistance and fall of compliance 5 and 10 min after aerosol delivery as compared to the baseline value. A one-way analysis of variance (one-way ANOVA) of these parameters was performed to show statistical difference between control and treated groups.

RESULTS

The nebulization screening study of the various cetrorelix solutions allowed the selection of four formulations for the aerosol delivery in rats (Table I). In these cetrorelix solutions, a positive effect on total aerosol output was shown by addition of Tween-80 or ethanol. Their effects were synergistic and the combination of both adjuvants improved the output. In contraposition, α-lecithin showed a positive effect only in presence of ethanol. After nebulization, no sign of substance degradation both in the collected nebulisate and in the remaining solution of the nebulization chamber was observed (data not reported) as shown in a previously study performed by the ASTA-ADS (13). The calculated MMAD of the nebulized formulation ranged between 4 and 5 μm, whereas the GSD ranged between 1.9 and 2. The determination of the MMAD was performed in our laboratories using a previously validated and routinely used method by a single determination. The values are reported in Table I.

After aerosol delivery in rats of the placebo and cetrorelix formulation by the ASTA-ADS, the testosterone concentration of all placebo and verum groups were measured and expressed as mean ± standard deviation (SD). For all cetrorelix-treated groups, a fall of testosterone level was evident 6 h after aerosol administration (Fig. 2). The antagonistic effect was extended up to 24 h after delivery. This difference was statistically significant as compared to the values of the placebo groups at the same sampling time (Table II). After this time, the level of testosterone returned to normal values and the treated groups did not show statistical difference to the placebo groups (Table II). The display of the pharmacologic effect after cetrorelix aerosol delivery was similar to the effect shown in a previous study after i.t. instillation of 0.5 mg/kg of cetrorelix (8). The mean value of the testosterone level and corresponding standard deviation for placebo groups, verum groups, and this i.t. instillation group are reported in Table II. The measurement of the cetrorelix concentration in both wash-bottles after aerosol delivery allowed calculation of the delivered as well as the deposited dose to the rat's lung (Table III). In the used dosing range (0.4–0.6 mg/kg), no statistical difference in the pharmacologic effect (testosterone concentration between 0.5 and 48 h, Table II) was observed between aerosol-treated groups and the i.t. group (0.5 mg/kg cetrorelix). Moreover, the obtained pharmacokinetic values from all four groups after determination of cetrorelix serum level by RIA did not show statistical difference both between aerosol treated groups as well as between aerosol groups and the i.t. instillation group with the exception of the $t_{1/2}$ and partially of the MRT (Table III). The pharmacokinetic parameters showed a MRT, and included between 7.4 ± 3.9 and 13.3 ± 6.1, a $t_{1/2}$ included between 17.9 ± 5.6 and 22.9 ± 8.0, and an absolute bioavailability between 16.4 ± 9.2% and 32.0 ± 19.0% for the delivered dose and between 48.4 ± 27.0% and 77.4 ± 44.0% for the deposited dose. A detailed summary of pharmacokinetics is shown in Table III.

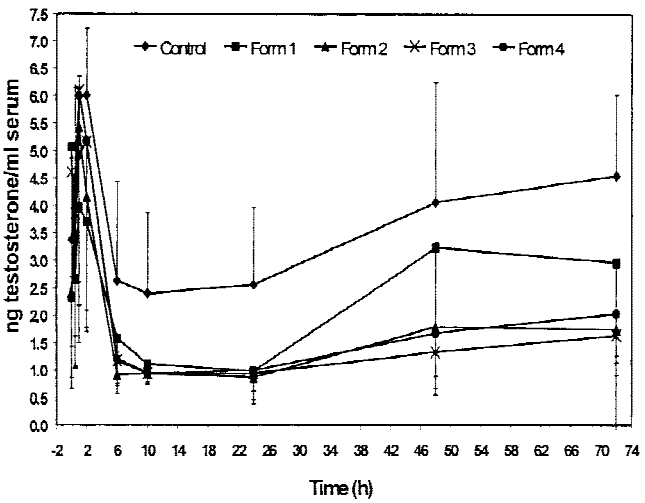

Fig. 2. Testosterone serum levels in rats after aerosol delivery of cetrorelix (form. 1 to 4, $n = 9$ each group) and of placebo solutions (mean of three placebo groups ($n = 6$ each group) receiving aerosol from isotonic saline, hypotonic solution of tween and α-lecithin, and isotonic mannitol solution, respectively). Error bars indicate mean value + SD (placebo) or − SD (verum). Numerical values are reported in Table II.

The histologic examination showed that animals that received formulation 3 or the three different placebo formulations did not develop any treatment-related findings in the lungs (Fig. 3). The vast majority of lung parenchyma was also unaffected after administration of formulations 2 or 4. However, individual terminal bronchioles and/or alveolar ducts contained minimal to moderate focal accumulations of amorphous chromophobic foreign material (Fig. 4, left). The finding occurred in an incidence of 4/6 rats treated with formulation 2 and 2/6 animals treated with formulation 4. The foreign material, which probably represents the test compound, was associated with a minimal to mild localized subacute inflammatory reaction. Formulation 1 induced more pronounced histopathologic lesions affecting entire lobes whereas others were unaffected. The predominating findings were a slight to severe alveolar edema, associated with alveolar histiocytosis and alveolar mixed inflammatory cell infiltrates (Fig. 4, right). These lesions indicate intrapulmonal intolerability of formulation 1.

The measurement of lung resistance and lung compliance during the aerosol administration of isotonic saline ($n = 9$), and the four cetrorelix formulations ($n = 6$ for form. 1–3 and $n = 5$ for form. 4) revealed no signs of treatment-related changes in lung function parameters. The minimal changes (fall of compliance down to –20%; rise of resistance up to 20%) are within the normal deviation range of the system, thus they are in the normal lung parameters range of automatically ventilated rats (Table IV) (24). Moreover, the one-way ANOVA comparing verum groups vs. placebo group does not show any statistical difference (Table IV). These results indicated that the aerosolization of cetrorelix formulations containing ethanol, Tween-80, and α-lecithin at the used concentration does not produce signs of local intolerability such as bronchospasm or alteration on the viscoelastic properties of the respiratory region.

DISCUSSION

Ultrasonic nebulization of solutions represent a simple and an efficient method for peptide-drug delivery especially by automatically ventilated subjects (25). Unfortunately, the theories on ultrasonic nebulization, especially the currently more accepted cavitation wave theory (17), are not adequately supported by mathematics and the influence of the various factors, such as the physicochemical properties of fluid (most important density, viscosity, and surface tension) and the operative condition, on the nebulization performance (output and particle size distribution) cannot exactly be foreseen (17,18). In this study, the density, viscosity, and surface tension of the formulations were measured (data not reported) to calculate particle size. Unfortunately, it was not possible to correlate the theoretical value with experimental data. However, we can affirm that the formulations giving a capillary wavelength (calculated by the cavitation-wave theory using the measured physicochemical parameters) more than 8.4 μm (e.g., 1.5 mg/ml cetrorelix in water) were incapable of being nebulized at 1.7 MHz. Thus, the development of appropriate formulations for ultrasonic nebulization must be performed experimentally. Although the aerosol formulations could present little deviation from physiologic to-

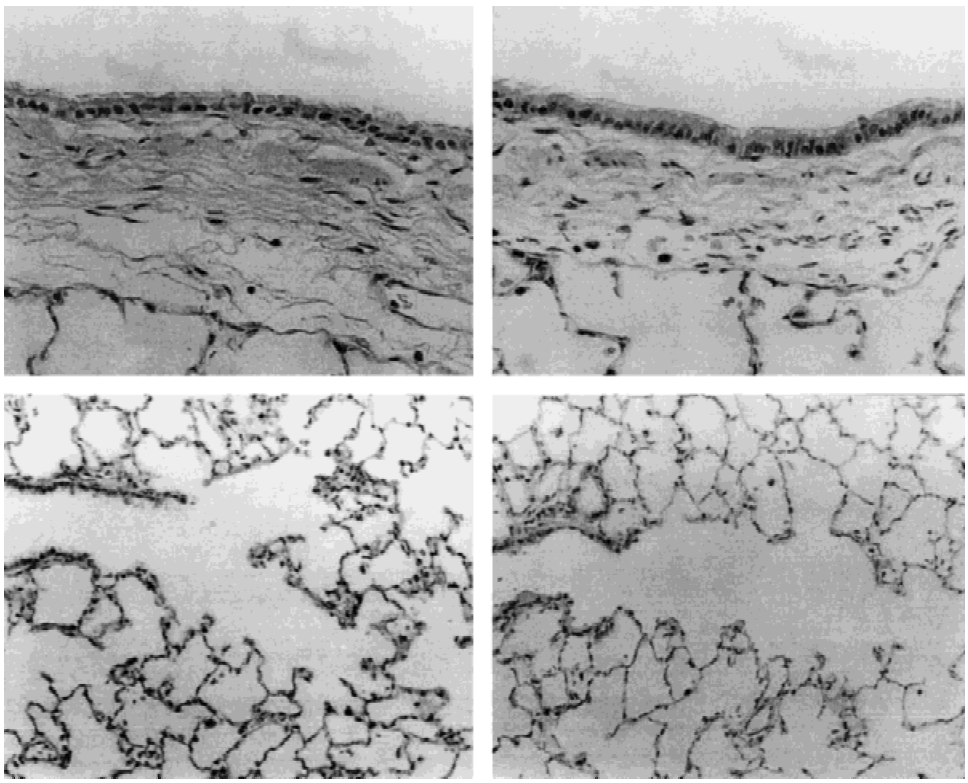

Fig. 3. Morphology of pulmonary tissue after intratracheal aerosol administration of cetrorelix in formulation 3 (left column); right column shows comparable regions of a control animal. Top: no morphologic effects on the bronchial epithelium (hematoxylin and eosin, 340×). Bottom: bronchioloalveolar region unaffected (hematoxylin and eosin, 170×).

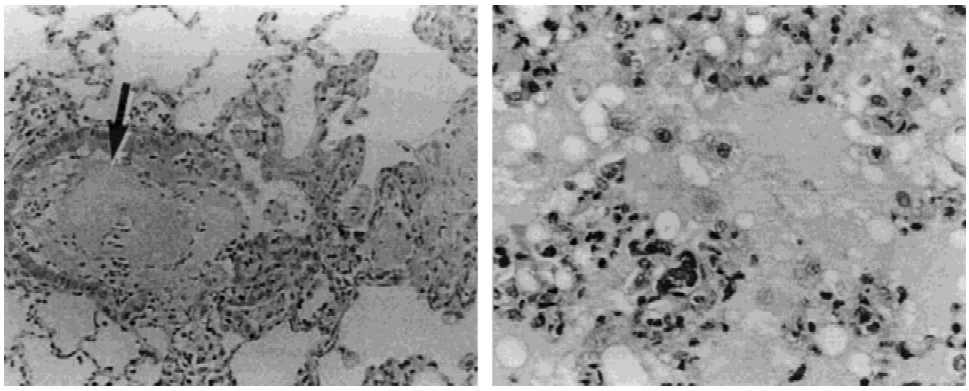

Fig. 4. Histopathology of the lung after intratracheal aerosol application of cetrorelix in formulation 2 (left) and 1 (right). Left: deposition of foreign material with reactive histiocytosis, fibroplasia, and minimal inflammatory cell infiltrates in individual bronchiolo-alveolar transitions after application of formulation 2; note also reactive hypertrophy of bronchiolar and alveolar epithelium adjacent to the foreign material (hematoxylin and eosin, 170×). Right: alveolar edema and histiocytosis induced by formulation 1 (hematoxylin and eosin, 340×).

nicity or physiologic pH, as demonstrated by various formulations for aerosol delivery on the market (e.g., Vividrin®, 43 mOsm; pH 5.6), the respiratory tract presents a very sensitive epithelium with numerous nervous terminations and deviation from tonicity and physiologic pH may result in bronchoconstriction and tissue damages. For the same reason, the use of additives, absorption enhancers, and/or protease inhibitors should be limited and examined case by case using adequate toxicologic studies. As observed in a previous study (8), cetrorelix passes through the pulmonary epithelium without the use of absorption enhancers or peptidase inhibitors. However, in this study, some adjuvant for nebulization was required to improve the aerosol output from the device.

Some authors affirmed that proteins undergo denaturation during ultrasonic nebulization (18). We did not observe cetrorelix degradation after ultrasonic nebulization, as shown by chromatographic analysis of the nebulized solutions and of the solution remaining in the nebulization chamber (data not shown), nor loss of activity as shown by the pharmacologic answers. Probably, some peptides oppose a low resistance against the ultrasonic waves and can be nebulized more easily than big proteins. These latter compounds possess frequently secondary and possibly tertiary structures, which may easily undergo denaturation even by physical denaturants, such as heat and mechanical shaking, both present by ultrasonic nebulization (17,18). Moreover, the protective action of the used adjuvant against denaturation during ultrasonic nebulization already has been demonstrated (17,18,25), and also may be responsible for the retained activity.

Evaluating the response of the animals to a systemic delivery of a therapeutic agent requires the knowledge of the amount of agent at the site of administration, in our case, the pulmonary epithelium (4). Moreover, quantification of the dose is essential for comparison of results among different investigators and laboratories. In previous studies performed with the ASTA-ADS, we observed a high precision of aerosol delivery, a complete recovery of nebulized substance (thus, the aerosol does not leak out of the system), and an elevated efficiency of the wash-bottles (thus, this procedure is reliable for capturing aerosol and therefore for calculating the deposited dose). Our system showed that it is capable of aerosol delivery in orotracheally cannulated rats, determining the delivered dose in an efficient and reproducible manner (13). Precision and accuracy of the mentioned delivery and analytical systems, such as ASTA-ADS, HPLC, ELISA, and RIA have been already described in our previous studies (8,13,21) and therefore is not repeated here. After aerosol delivery of cetrorelix to rats by the ASTA-ADS, we noted that the obtained pharmacologic and pharmacokinetic parameters were similar to those obtained in a previous study by i.t. instillation

Table IV. Determination of Lung Resistance and Dynamic Compliance After Aerosol Delivery of the Four Cetrorelix Formulations by Pulmonary Monitoring System as Compared to the Placebo Group (Isotonic Saline)

	Placebo (n = 9) mean (SD)	Form. 1 (n = 6) mean (SD)	Form. 2 (n = 6) mean (SD)	Form. 3 (n = 6) mean (SD)	Form. 4 (n = 5) mean (SD)	SD one-way ANOVA
R_L (%) after 5 min	3.5 (5.8)	9.1 (5.0)	11.7 (12.4)	10.2 (10.6)	6.4 (7.4)	NO $P = 0.379$
R_L (%) after 10 min	0.9 (4.8)	5.4 (4.4)	10.0 (14.5)	8.7 (8.6)	5.1 (7.6)	NO $P = 0.291$
C_{dyn} (%) after 5 min	–9.4 (4.3)	–9.0 (5.4)	–13.6 (10.0)	–7.5 (3.2)	–8.0 (6.8)	NO $P = 0.473$
C_{dyn} (%) after 10 min	–10.8 (4.4)	–11.1 (5.4)	–15.4 (11.5)	–10.4 (4.9)	–11.7 (7.4)	NO $P = 0.747$

of 0.5 mg/kg of cetrorelix in rats (8). A statistical examination of these parameters, such as testosterone level, AUC, and C_{max} between the i.t. instillation group and aerosol groups confirmed this observation (Tables II and III). It must be noted that the drop of the testosterone serum level observed in all placebo groups, most probably related to the anesthesia procedure (8,13), gives results statistically different from the testosterone serum level observed in the verum groups. The calculation of the corrected dose, by subtraction of the exhaled amount from the delivered amount of substance, using the formula in Fig. 1, allowed us to explain these similarities. The corrected dose of three delivered aerosol formulations were not statistically different from the delivered dose by i.t. instillation. For the calculated deposited dose of the one formulation, a statistical difference at the used probability level ($P = 0.05$) cannot be excluded. However, the statistical examination of the absolute bioavailability values, calculated by the corrected dose, did not show a statistical difference between groups, also demonstrating, together with the other observations, the validity of the ASTA-ADS and of the use of the corrected dose. As reported in Table III, the difference between delivered and deposited dose is remarkable. The exhaled dose always should be measured in opposition to the most aerosol delivery studies in which the exhaled dose is ignored. However, a better determination of droplets size distribution of the aerosol application should be performed. The aerosol flow used for the determination of the MMAD (30 l/min) was different from the flow of aerosol used for the application (1.68 l/min) because of limitations of the analytical method and of the respiratory parameters of the animals, respectively. Therefore, the value of particle size distribution should be considered as indicative for an eventual application in humans and not as effective droplets size applied to animals.

The reason for using mannitol in a placebo group was to compare it with the i.t. instillation, whereas the use of the medium of formulation 4 was to show eventual influence of the adjuvants (all present in the medium of formulation 4) and/or of the hypotonic solution. Because all placebo applications did not show any pharmacologic and toxicologic effect, they have been associated in a single group for a better statistical significance.

The microscopic examination revealed that formulation 3 (i.e., cetrorelix in an aqueous solution with ethanol and α-lecithin), did not induce histologic lesions of the lung parenchyma. Formulations 2 and 4, containing a combination of ethanol and Tween-80, gave rise to focal accumulations of foreign material, which probably represents the test material. This is suggestive of an uneven intrapulmonal distribution of cetrorelix in these formulations. The predominantly histiocytic infiltrates associated with the foreign material represent a physiologic reaction indicating its removal by phagocytosis. Therefore, the slight inflammatory cell infiltrates as well as fibroplasia and hyperplasia of type II alveolar epithelial cells have to be interpreted as secondary and not as directly treatment related (26). Formulation 1, which did not contain ethanol and α-lecithin, induced pronounced parenchymal lesions in the lung, indicating local intolerability. The absence of mechanical lung lesions like emphysematous processes in all treatment groups including control subjects indicates that the experimental parameters were adequately chosen and guarantee physiologic conditions for the artificial mechanical ventilation. Moreover, no signs of bronchoconstriction or other alteration of lung function during aerosol delivery were observed, as demonstrated by measurement of the lung resistance and compliance. Lung resistance is the sum of the tissue resistance, offered by the viscoelastic properties of the parenchyma and alveolar lining, and the airway resistance, that is, the resistance to air flow due to friction in the airways (27). The lung compliance (C_L) is a measure of the ease of lung inflation, that is, the "stretchability" of the tissue, and is markedly affected by the elastic nature of the lung parenchyma and surface tension forces in the alveoli (27). Dynamic compliance (C_{dyn}) is measured during respiration when resistance to air flow may affect the value. Using both pulmonary and histologic monitoring, the presence, site, and extent of pulmonary disease and alteration thus can be estimated (24). However, further toxicologic studies should be performed for a better understanding of the intolerability of form. 1. It is important to note that only this formulation showed substance-related damage, whereas the other formulations, even containing Tween-80 (i.e., form. 2, form. 4, and one placebo solution utilized as a control solution), did not show such alteration. Further studies could be performed for giving an interpretation of these differences. For example, Broncho-Alveolar Lavage (BAL) fluid could be studied for determining enzyme, cytokine, and reactive intermediate concentration (12,26). Additionally, because Tween-80 is yet not approved for use in pulmonary delivery, toxicologic studies in other nonrodent species should be performed. Given the surface-active environment into which the cetrorelix formulations are being delivered, it seems unlikely that the utilized concentration of Tween-80 (≈375 μM) could enhance absorption. The results are in general agreement with other investigations that have used surface-active ingredients by the pulmonary application of peptides and proteins. Niven *et al.* (19) did not observe pulmonary absorption enhancement of a test peptide in rats, after aerosol application of a peptide aerosol containing Tween-80 (75 μM). Even 20 mM concentration of the surfactant in another similar experiment have been shown to only marginally improve the pulmonary absorption of insulin, as shown by Li *et al.* (28). However, the use of alveolar epithelial cell monolayers could be an adequate way for studying this aspect (29).

CONCLUSIONS

Efficient systemic pulmonary delivery of the peptide drug cetrorelix to rats could be demonstrated using a specially designed aerosol delivery system (ASTA-ADS). This study emphasizes the importance of precisely determining the delivered and exhaled dose of aerosol to calculate bioavailability and other pharmacokinetic parameters. Based on our results, cetrorelix seems a promising candidate for systemic delivery as an inhalation aerosol, and deserves further evaluation in humans.

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