

Clinical Trials

Phase I and Pharmacokinetics Study of Crotoxin (Cytotoxic PLA₂, NSC-624244) in Patients with Advanced Cancer¹

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ABSTRACT

A Phase I clinical trial was performed on patients with solid tumors refractory to conventional therapy. Crotoxin was administered i.m. for 30 consecutive days at doses ranging from 0.03 to 0.22 mg/m². Patients entered the study after providing a written informed consent. Although 26 patients were entered only 23 were evaluated. Reversible, nonlimiting neuromuscular toxicity evidenced as diplopia because of pareses of the external ocular muscles was present in 13 patients. It started at doses of 0.18 mg/m² and lasted from 2 to 6 h. These episodes did not require dose adjustment and disappeared in 1–3 weeks of treatment. Three patients experienced palpebral ptosis, nystagmus (grade 2), and anxiety (grade 2–3) at the dose-limiting toxicity of 0.22 mg/m². Also at dose-limiting toxicity, 1 patient showed nystagmus (grade 2) and anxiety (grade 3) without evidence of palpebral ptosis. Transient increases (grades 1–3) in the levels of creatinine kinase, aspartate aminotransferase, and alanine transaminase attributed to crotoxin myotoxicity were observed but returned to normal by the last week of treatment. At 0.21 mg/m² there was a case of grade-3 anaphylactic reaction on day 31, which required treatment. Hypersensitivity was regarded as an adverse drug-related reaction, and the patient was removed from the protocol. Two patients at different doses (0.12 mg/m² and 0.22 mg/m²) had sialorrhea. Four patients had asymptomatic transient increase in blood pressure (up to 20 mm Hg) 12 h after the first injection, which lasted 24 h. No treatment was required and toxicity did not reappear. Six patients experienced slight eosinophilia during the first 2 weeks. The maximum tolerated dose was set at 0.21 mg/m². Objective measurable partial responses (>50% reduction of tumor mass) were noted in 2 patients treated at 0.21 mg/m² and 1 at 0.12 mg/m². One patient (at 0.21 mg/m²) presented a complete response on day 110. Crotoxin pharmacokinetics showed rapid absorption from the injection site to blood ($t_{1/2}$

$t_{1/2\alpha} = 5.2 \pm 0.6$ min). Plasma concentration reached a peak ($C_{\max} = 0.79 \pm 0.1$ ng/ml) at $t_{\max} = 19 \pm 3$ min. The half-life of the distribution (α) phase is 22 ± 2 min. Starting at 1.5 h after injection, the decrease in plasma concentration becomes slower, reaching 14 ± 3 pg/ml 24 h after injection. The profile is dominated by the elimination (β) phase with a half-life of 5.2 ± 0.6 h. Consequently, 24 h after the injection (~ 5 half-life) 97% of the product was eliminated. The area under plasma concentration *versus* time curve was 0.19 ± 0.05 $\mu\text{g}/\text{min}/\text{ml}$. Assuming availability (F) ~ 1 , the clearance is $C_L = 26.3 \pm 7$ ml/min, and the apparent volume of distribution is $V_d = 12 \pm 3$ liter/kg. The recommended dose for a Phase II study is 0.18 mg/m².



INTRODUCTION

Crotoxin is a cytotoxic PLA₂³ compound isolated from a South American snake, *Crotalus durissus terrificus*, venom. It is a noncovalent complex formed by two nonidentical subunits, one acidic (subunit A ~ 9.5 kDa) and one basic (subunit B ~ 14.5 kDa). Subunit B is a PLA₂ formed by a single chain of 122 amino acid residues cross-linked by seven disulfide bonds (1, 2, 3, 4, 5, 6). Subunit A is formed by three polypeptide chains cross-linked by seven disulfide bonds (7), is devoid of catalytic activity, has no affinity for membranes, and is nontoxic (LD₅₀ i.v. mice > 20 mg/kg; Ref. 6). The two subunits form spontaneously a tight 1:1 complex (6, 8). Complex formation inhibits the PLA₂ activity but increases toxicity by at least one order of magnitude (6, 9, 10, 11). Two mechanisms, acting synergistically, may explain this phenomenon: (a) subunit A acts as a "chaperone" preventing the nonspecific binding of subunit B to membranes (2, 6, 12); and (b) the specific binding of ¹²⁵I-crotoxin to target membranes (13, 14) suggests that subunit A may be involved in target recognition. Thus, crotoxin circulates nondissociated (*i.e.*, as a complex) until it recognizes specific "acceptor sites" on the target membranes. Some of these sites have been identified (15, 16). On binding, crotoxin dissociates into their subunits. Subunit B remains bound, whereas the subunit A is released to the medium (2, 17). Thus, subunit A transforms the PLA₂ from an unspecific cytotoxin into a self-target toxin compound.

Crotoxin displays cytotoxic activity against a variety of murine (4) and human tumor cell lines *in vitro* (18), which require both the PLA₂ activity of subunit B (4) and the ability of the complex to dissociate into their subunits (4, 19). Crotoxin-induced cytotoxic effects appear to be highly selective toward cell lines expressing a high density of epidermal growth factor receptors (19), thus suggesting that epidermal growth factor receptors, or a receptor function, play a role in targeting. On binding and subsequent crotoxin dissociation, the bound subunit B starts a rapid phospholipid hydrolysis around the "acceptor site," (a highly localized and/or specialized event as proposed for myotoxic PLA₂ (20, 21), which leads to cell death. Antitumor efficacy *in vivo*, using daily i.m. administration of crotoxin, has been demonstrated on Lewis lung carcinoma (83% growth inhibition; Ref. 22) and MX-1 human mammary carcinoma (69% growth inhibition). A lower activity (44% growth inhibition) was observed with HL-60 leukemia cells, suggesting that crotoxin may have a certain specificity toward solid tumors.

Crotoxin produces a limited number of toxic effects. Neurotoxicity is the most salient and consists of a peripheral blockade of neuromuscular transmission (23, 24, 25). In addition, when administered i.m., crotoxin showed myotoxic activity (26, 27). Crotoxin-induced myonecrosis is followed by muscle regeneration, which successfully resolves within 3 weeks (27, 28). Preclinical studies on acute toxicity using sublethal crotoxin doses evidenced paresis because of incomplete neuromuscular blockade, which were fully reversible (29).

Pharmacokinetic studies in mice showed that after i.m. administration of 0.1 mg/kg crotoxin, plasma concentrations fell from 25 to ~12 ng/ml in 160 min. This represents 3–1.5% of the IC₅₀ values, and, in accordance with previous studies on organ distribution of ¹²⁵I-crotoxin (9), >90% of the toxin is eliminated in 24 h. Therefore, a schedule of daily administration is viable. Subchronic toxicity studies on mice over a 30 day period, using daily i.v. doses of crotoxin up to 0.04 mg/kg, showed paresis of the rear limbs during the first week of treatment, which disappeared thereafter, perhaps because of a tolerance mechanism. Studies on blockage of phrenic nerve-diaphragm preparations from treated mice required crotoxin concentrations 10–20-fold higher than those for untreated mice (29). Subchronic i.m. toxicity studies on beagle dogs (30 day daily administration) also indicated a progressive decrease in crotoxin-induced myotoxic effects. The increase in plasma levels of CK, AST, and ALT released from the injured muscular cells peaked on study day 6 and then decreased progressively to normal levels on study day 20, even after continued treatment and rotated sites of injection (30). No hematopoietic, hepatic, or renal toxicities were observed in these studies (30).

The primary objectives of the present study were to identify the DLTs, establish the MTD, and obtain the pharmacokinetic profile for crotoxin when administered i.m.

► MATERIALS AND METHODS

Eligibility.

Patients entered had histologically confirmed solid tumors not treatable by surgery, radiation therapy, or standard chemotherapy. Eligibility criteria included the following:

(a) age ≥ 18 years; (b) an ECOG performance status of ≤ 2 and with reasonable nutritional state as to maintain their body weight; (c) a life expectancy of ≥ 12 weeks; (d) no recent major surgery (≤ 21 days); (e) no previous chemotherapy within the previous 4 weeks and recovery from any reversible toxic effects of previous treatments; (f) no previous radiation therapy for at least 6 weeks; and (g) adequate hematopoiesis (neutrophil count $\geq 1,500/\text{mm}^3$; platelet count $\geq 150,000/\text{mm}^3$; hemoglobin level ≥ 9 g/dl), renal (serum creatinine ≤ 2 mg/dl), and liver function (AST and ALT ≤ 3 x institutional upper limit of normal and serum bilirubin within institutional upper limit of normal). Regarding concurrent therapies, patients should be on stable doses of any drugs that may affect hepatic metabolism or renal drug excretion (e.g., analgesics, nonsteroid anti-inflammatory drugs, narcotics, and probenecid). Such drugs should not be initiated while patients are participating in the study. Exclusion criteria included severe allergy and hypersensitivity to crotoxin, pregnancy, lactation, active or

uncontrolled infections (including AIDS); symptomatic interstitial fibrosis of the lung causing \geq grade 2 dyspnea; preexisting peripheral neuropathy; diabetes mellitus; frequent vomiting/poor alimentation; recent history of weight loss ($>10\%$ of current body weight); and any other medical condition severe enough as to prevent full compliance with the study protocol.

Twenty six patients were enrolled after giving a written informed consent, according to institutional and federal guidelines, before treatment. Four patients had breast cancer, 7 patients had gastrointestinal cancer, 3 patients had non-small cell lung cancer, 2 patients had squamous cervix carcinoma, 2 patients had prostate cancer, and 1 patient each had thyroid carcinoma, larynx carcinoma, bladder carcinoma, Fallopian tube adenocarcinoma, head and neck cancer, low grade fibrosarcoma, Ewing's sarcoma, and liposarcoma.

Treatment Plan.

Crotoxin was administered daily i.m. for 30 consecutive days. One patient was studied at the dose level of 0.03 mg/m^2 (i.e., $1/20$ of the LD_{10} i.m. in mice) to search for unexpected toxicities. No toxicity occurred after 3 weeks of treatment. Three patients were enrolled at each dose level and observed for at least 3 weeks before enrolling any patient at the next dose level. Planned dose escalations were 0.06 mg/m^2 , 0.12 mg/m^2 , and 0.18 mg/m^2 . Additional increases in dose levels were smaller: 0.21 mg/m^2 and 0.22 mg/m^2 . If DLT was observed in 1 of the 3 patients enrolled at a given dose level, 3 additional patients were enrolled at the same level. DLT in ≥ 2 patients identified that as the DLT dose level. The MTD was defined as one level below the DLT dose level. After identification of the MTD, additional patients were enrolled to more fully evaluate the toxicities at MTD. DLT was defined as: (a) grade 2 neural toxicity characterized by incoordination (23, 30), nystagmus (23), and grade 2–3 anxiety occurring for ≥ 2 consecutive days; and (b) any other toxicity grade 3 intensity or higher. Toxicities were graded according to the National Cancer Institute Common Toxicity Criteria. Inpatient dose escalation was not permitted in this study. Because preclinical studies showed that the i.m. injection of crotoxin produced increased plasma levels of CK, AST, ALT, and lactic dehydrogenase because of muscular toxicity, increases in plasma levels of AST and ALT up to 5 x institutional upper limit for normal (grade 2) during the first 2 weeks of treatment were allowed if not accompanied by other signs of hepatic toxicity.

Crotoxin.

Dried *C. durissus terrificus* venom was obtained from "Centro Zootoxicológico Misiones" (Oberá, Misiones, Argentina). Purification of crotoxin and isolation of the subunits were performed as described previously (22). Amino acid composition and NH_2 -terminal analysis of both subunits were consistent with data published previously. The isolated subunit B had a PLA_2 -specific activity of 76 ± 6 units/mg protein (pH-Stat) and an LD_{50} (i.v., mice) of $0.56 \pm 0.12 \text{ } \mu\text{g/g}$. No enzymatic activity or toxicity was observed associated with subunit A. The reconstituted crotoxin complex gave a single peak ($24 \pm 0.2 \text{ kDa}$) by analytical gel filtration on a prepacked Superdex 75 HR column, and had a PLA_2 -specific activity of 21 ± 3 units/mg protein and an LD_{50} (i.v., mice) of $0.06 \pm 0.015 \text{ } \mu\text{g/g}$. Analysis by SDS-PAGE (PhastGel 8–25% gradient) under nonreducing conditions (silver stain) showed two bands with molecular weights $9.5 \pm 0.3 \text{ kDa}$ (subunit A) and $14.5 \pm 0.2 \text{ kDa}$ (subunit B). The LD_{10} i.m. in mice was estimated at $0.19 \text{ } \mu\text{g/g}$ (95% CI, 0.13–0.23). Crotoxin samples were dissolved in sterile,

pyrogen-free PBS, sterilized by filtration, diluted to a final concentration of 2.0 ± 0.08 mg/ml, and fractionated into 1-ml vials. Acceptability criteria on 10% of the vials were tested independently by the National Institute of Medicines, Buenos Aires, Argentina (INAME, Instituto Nacional de Medicamentos).

Pretreatment and Follow-Up Evaluation.

Prestudy evaluations comprised a complete history and physical examination including height and weight, ECOG performance score, complete blood count, serum electrolytes and chemistries, tumor markers (when appropriate), urinalysis, chest X-ray, and electrocardiogram. Serum pregnancy tests were performed in women with childbearing potential. Whenever the patients had objectively measurable lesions, their location, number, and baseline dimensions were recorded by computer-assisted tomography scan before and at the end of each 30-day cycle. Study parameters such as physical examinations and expected toxicities were recorded daily. FVC was measured daily, 2–3 h after crotoxin injection for the first 2 weeks, and neurological examinations were performed as required. Laboratory studies, including hematological, clinical chemistry, and urinalysis were performed weekly. At the end of a 30-day cycle, patients were allowed to receive protocol therapy for up to 2 additional 30-day cycles without an interval between cycles, unless progressive disease was discovered, intercurrent disease prevented additional treatment, or the patient (or patient physician) decided to discontinue the treatment.

Pharmacokinetics.

Blood samples from 5 patients were obtained before and up to 24 h after the i.m. injection of crotoxin at 0.21 mg/m^2 on study days 1, 15, 30, 60, and 90.

Sample Collection.

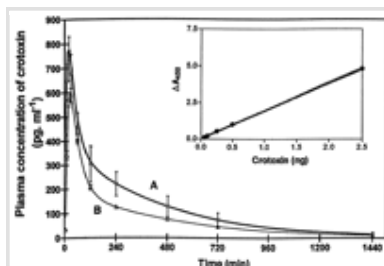
Blood samples (1 ml) were drawn through an indwelling i.v. cannula and discarded. Then 9-ml samples were collected into heparin-containing tubes before and 5, 10, 20, 30 min, 1, 2, 4, 8, 12, and 24 h after the i.m. injection of crotoxin. Collection tubes were immediately placed into a slurry of ice water. The plasma was separated by centrifugation ($1200 \times g$) for 20 min, transferred to sterile, labeled, plastic tubes, and stored at -30°C until assay. The time of storage was within 6 months, supported by stability data.

Assay Methods.

Horse IgG against *C. durissus terrificus* venom and the IgG fraction from serum of New Zealand rabbits immunized with crotoxin were provided by Instituto Nacional de Producción de Biológicos A.N.L.I.S. "Carlos G. Malbrán." The IgG fractions were additionally purified in prepacked protein G-Sepharose (MABTrap) columns (Pharmacia).

The anticrotoxin IgGs were purified by immunoaffinity chromatography on a crotoxin-NHS-Sepharose column (5 ml) prepared as described by manufacturers. "Double sandwich" ELISA assays amplified by peroxidase were performed on plates sensitized with $2 \mu\text{g}$ of immunopurified anticrotoxin horse IgG. This method was used to determine crotoxin concentration in plasma and urine. However, crotoxin concentrations in plasma fell below the sensitivity limit of this assay (0.3 ng/ml) 1 h after injection, and another method was required. Samples of patient plasma or normal human plasma containing $0.05\text{--}2.5 \text{ ng}$ of added crotoxin (calibration curve) adjusted to 5 ml with PBS

containing 1 mg/ml BSA were applied to 1 ml prepacked NHS-Sepharose (HiTrap) columns coupled with 2 mg of horse anticrotoxin-IgG. Unbound material was washed. Then, 5 ml samples containing: (a) 0.2 mg/ml immunoaffinity purified rabbit anticrotoxin IgG; (b) 0.1 mg/ml of biotinylated goat antirabbit IgG (Sigma Chemical Co.); and (c) 0.1 mg/ml avidin-peroxidase conjugate (Sigma Chemical Co.) were sequentially applied to the column, each addition followed by a wash. The immune complexes plus the bound avidin-peroxidase conjugate were eluted with 0.1 M sodium formate buffer (pH 2.5; fractions collected on 1 M sodium phosphate buffer and adjusted to pH ~5.2). Peroxidase activity in the eluate was determined on samples diluted in 50 mM phosphate-citrate buffer (pH 5.0) equilibrated at 25°C by the increase in A_{450} nm after the addition of 5.5 mM *o*-phenylene diamine (Sigma Chemical Co.) and 3 mM hydrogen peroxide. "Blank" eluates gave ΔA_{450} values of <0.001/min. Five or six samples with different volumes of each eluate were measured to ensure linearity between volume and ΔA_{450} /min. The minimum acceptable values of ΔA_{450} was 0.015/min. As shown in Fig. 1 (inset) the plot of total ΔA_{450} [*i.e.*, ΔA_{450} /min (\pm SD)] \times total volume of eluate is a straight line. $R^2 = 0.999$ with a slope 1.913 (95% CI, 1.874–1.973) ΔA_{450} /min/ng crotoxin. The calculated sensitivity limit was 30 (95% CI, 14.5–76.1) pg crotoxin. The coefficient of variation interassay for a single column was 4.5% and for different columns was 12.5%. Urine was collected at the intervals 0–2, 2–4, 4–6, 6–8, 8–12, and 12–24 h after injection and placed into labeled sterile flasks. The volume was recorded, and 10 ml were sterilized by filtration. The samples were diluted 1:10 with sterile PBS (except for the last sample, which was diluted 1:5) and kept into sterile plastic tubes at -20°C until assay. Crotoxin concentration was assayed by "double sandwich" ELISA on plates sensitized with 2 μ g of immunopurified anticrotoxin horse IgG as described above. Plasma from patient blood samples obtained before daily injection of crotoxin were used for detection and quantification of anticrotoxin antibodies. ELISA plates were sensitized with horse anticrotoxin IgG and blocked as described previously. The final volume of all of the components was 100 μ l. Incubations were performed for 60 min at 30°C followed by five washes. The wells were incubated sequentially with: (a) crotoxin, 10.0 ng/ml; (b) a 1/10 dilution of normal human plasma (negative controls) or plasma samples from patients; (c) rabbit anticrotoxin IgG 40 ng/ml; (d) biotinylated goat antirabbit IgG; (e) avidin-peroxidase conjugate; and (f) peroxidase substrate mixture. The reaction was measured as described above. Using normal human plasma the A_{492} was $\sim 1.0 \pm 0.1$. When plasma samples contained anticrotoxin antibodies the A_{492} was $< 1.0 \pm 0.1$. In such cases the ELISA was performed using biotinylated goat antihuman IgG (Sigma Chemical Co.), avidin-peroxidase conjugate, and then with peroxidase substrate mixture.



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Fig. 1. Plasma concentration *versus* time curves of crotoxin after the i.m. administration to patients at the dose level of 0.21 mg/m². Curve A, the points represent means obtained from five patients on study day 1; bars, \pm SD. The points were fitted to the pharmacokinetic equation: $C_t = 1.11 \times \exp(-0.0315 \times t) + 0.38 \times \exp(-0.002 \times t) - 1.53 \times \exp(-0.112 \times t)$ where C_t is the crotoxin concentration in ng/ml at time t ; the constants are expressed in

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ng/ml, and the exponential coefficients represent the rate constants for the distribution, elimination, and absorption phases, in that order, expressed in min^{-1} . *Curve B*, data obtained for a patient after the i.m. injection of crotoxin at the dose level of 0.21 mg/m^2 on study day 30. *Inset*, calibration curve for determination of crotoxin. The change in absorbance ($\Delta A_{450}/\text{min/ml} \times \text{total volume of the eluate}$) was plotted as a function of the amount of crotoxin added to the sample. The results are presented as mean \pm SD obtained from three different immunoaffinity columns. Linear regression analysis indicates: slope = ± 0.013 ; intercept = 0.0318 ± 0.0153 ; and $r^2 = 0.998$. *Dotted lines*, the limits of 95% CI.

Data Analysis.

Except for the 24 h sample, three to five values of crotoxin concentration in plasma were obtained for each time point, and the results were expressed as mean \pm SD and introduced in an archive of a diskette. The values of C_{max} and τ_{max} were determined from individual patients concentration-time curves. The AUC values were determined using the linear trapezoidal rule from time 0 to the last sampling time at which quantifiable concentrations of crotoxin were detected. The apparent terminal phase of elimination was determined to be in the terminal log-linear region of the plasma concentration-time curves. Fitting to compartment model and statistical comparison between models was performed using the combined Prism-StatMate softwares (GraphPad Software, Inc., San Diego, CA).

▶ RESULTS

Distribution of patients according their age, sex, performance status, primary disease, and stage is shown in Table 1*. Three of the entered patients could not be evaluated. A 46-year-old female with stomach cancer presented abdominal pain and jaundice because of biliary obstruction on study day 6 and required surgery. Another patient died on day 3 because of pulmonary edema unrelated to drug treatment. A 69-year-old male with prostate cancer was unable to adapt to inpatient study conditions and abandoned the protocol on study day 8. Thus, a total number of 35 cycles of crotoxin administration were evaluated in 23 patients. No deaths attributable to crotoxin toxicity occurred in this study.

View this table: [Table 1 Patients characteristics^a](#)
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Neurological Toxicity.

No significant toxicity was observed up to the dose level of 0.12 mg/m². At dose levels 0.18 and 0.21 mg/m² the most conspicuous symptom was grade 2 diplopia (usually horizontal or mixed) starting 2–4 h after injection and lasting 4–12 h (Table 2)*. Almost all of the patients reported diplopia, and it commonly appeared intermittently before study day 10 and fully disappeared on study days 15–21. Neurological examination indicated that it resulted from paresis of the external ocular muscles, impairing the fine adjustment required for image convergence. In comparison, the intrinsic ocular musculature appeared unaffected, and pupillary responses to light and accommodation were conserved. At a dose level of 0.22 mg/m² diplopia was at times accompanied by strabismus and/or palpebral ptosis. At this dose level 2 patients presented grade 2 incoordination and nystagmus episodes accompanied by anxiety (grade 3) for 2 consecutive days. Although these signs disappeared without any dose adjustment on continuation of the treatment, there was no additional dose escalation. Thus, the MTD was set at 0.21 mg/m². Neurological follow-up showed that paresis were self-limited to external ocular muscles and did not progress involving pharyngeal, laryngeal, or respiratory muscles before eventually disappearing. Clinical signs of dysphonia, dysphagia, or dysarthria were consistently absent. Except for 2 patients (1 having a tracheostomy and another with a previous maxillary surgery, which prevented proper performance of the test), measurements of FVC (2–3 h after crotoxin administration) showed that only 7 patients had ≤20% decrease in maximal respiratory pressure without clinical signs during the first week of the treatment. Significant decreases in FVC were observed in 8 patients, attributable to complications of the primary disease (*i.e.*, pleural effusion) or concurring infections.

View this table: Table 2 Neurotoxicity in patients treated with crotoxin

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Nonneurological Toxicities.

Nonneurological toxicities are presented in Table 3*.

View this table: Table 3 Non-neurological toxicity

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A 58-year-old female with cervix cancer treated at dose level of 0.21 mg/m² had intermittent grade 1 diarrhea lasting 24 h every 2–3 days during the first 2 weeks of treatment. This patient had a grade 3 anaphylactic reaction on day 31, which required treatment. Hypersensitivity was regarded as an adverse drug-related reaction, and the patient was removed from protocol. Two patients (a 68-year-old male with larynx cancer, treated at 0.12 mg/m², and a 58-year-old male with lung cancer, treated at 0.22 mg/m²) had sialorrhea during the study. Four patients had asymptomatic transient increase in blood pressure (up to 20 mm Hg) 12 h after the first injection, which lasted 24 h. No treatment was required, and it did not reappeared during the study.

Hematological studies did not show evidences of hemopoietic toxicity, and only in 6 patients (see Table 2*) was a transient eosinophilia observed during the first 2 weeks of treatment. Starting at 0.12 mg/m² all of the patients had grades 1–2 increases in the plasma levels of CK, AST, and ALT during the first 2 weeks of treatment, which returned to normal levels during week 4. These transitory increases in plasma levels of CK, ALT, and AST were not accompanied by alterations of liver functions, as indicated by levels of prothrombin, activated prothrombin time, plasma proteins, bilirubin concentration, and alkaline phosphatase. Six patients presented a slight increase in the levels of γ -glutamyl-transpeptidase, which could be attributed to the primary disease. Renal functions were not affected as shown by creatinine and urea concentrations as well as by urinalysis. No changes were observed in other metabolic parameters such as glucemia, uricemia, calcemia, or magnesemia.

Pharmacokinetics.

The plasma concentration-time course of crotoxin up to 24 h after the i.m. administration of 0.21 mg/m² on study days 1 and 30 is shown in Fig. 1*. The toxin was rapidly absorbed from the site of injection ($t_{1/2A} = 5.2 \pm 0.6$ min) to the blood. Crotoxin concentration in plasma reached a peak ($C_{max} = 770 \pm 110$ pg/ml) at $t_{max} = 19 \pm 3$ min and then dropped progressively. The changes in plasma concentration are best described by a two-compartment open model with a first-order absorption. The half-life of the distribution (α) phase is 22 ± 3 min and that of the disposition (β) phase is 5.2 ± 0.6 h. Assuming that availability (F) of crotoxin after i.m. injection is close to 1, clearance (CL) is 25 ± 8 ml/min, the AUC is 0.19 ± 0.05 μ g min/ml, and the apparent volume of distribution (V_d) is $\sim 12 \pm 3$ liters/kg. A summary of the pharmacokinetic parameters on study days 1 and 15 is presented in Table 4*. Crotoxin is also excreted by urine (Table 5)*. The cumulative amount of crotoxin "excreted unchanged" (*i.e.*, by ELISA assay) by 24 h after administration accounts for $7 \pm 2\%$ of the dose. Renal clearance (CL_R) of crotoxin was estimated as 1.8 ± 0.5 ml/min from the slopes of the amount of crotoxin excreted in urine within a collection interval *versus* the AUC within the same time interval.

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Table 4 Pharmacokinetic parameters of crotoxin administered i.m. to patients at the dose of 0.21 mg/m² on study days 1 and 15

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View this table: [Table 5](#) Urine data following the i.m. injection of crotoxin (0.21 mg/m²)
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As shown in Table 4*, most of the pharmacokinetic parameters of crotoxin on study day 15 are not significantly different than those on day 1. However, the peak concentration ($C_{\max} = 0.95 \pm 0.12$ ng/ml) seems to be higher and appears sooner ($t_{\max} = 15 \pm 2$ min). The half-life of the distribution (α) phase is 18.2 ± 4 min and that of the disposition (β) phase is 4.25 ± 0.3 h, both shorter than for day 1. The AUC and clearance are similar to those in day 1, whereas the apparent volume of distribution was smaller, *i.e.*, (V_d) = 9.1 ± 4 liters/kg.

Changes in plasma concentration *versus* time curves on study day 30 (Fig. 1)* consisting in an apparent decrease in the absorption rate ($K_A = 0.07 \pm 0.02$ min⁻¹) showed a lower peak of plasma concentration ($C_{\max} = 0.6 \pm 0.3$ ng/ml) appearing later ($t_{\max} = 23 \pm 3$ min) and with a smaller AUC (0.14 ± 0.07 µg/min/ml), possibly ascribed to the presence of anticrotoxin antibodies.

Response of Pain.

Eighteen of 23 evaluable patients reported a progressive but significant decrease or disappearance of pain (*e.g.*, associated to bone metastasis or distension) starting on the second or third week of treatment. This effect did not show daily fluctuations or decreased on continuation of the treatment. Pain decrease was assessed by reduction in consumption of analgesics and input of the patient.

Response of Disease.

The presence of objectively measurable lesions was not included as requirement for patient eligibility in this study. Four patients showed no progression of the disease. A 66-year-old man with a colon carcinoma and hepatic metastases, treated at 0.06 mg/m² crotoxin, had a 40% reduction of the hepatomegalia during a 60-day period. Thereafter, disease progression was reported. A 47-year-old man with a fibrosarcoma diagnosed by surgery and pelvic invasion, which produced an important edema in both legs, was treated with 0.21 mg/m² crotoxin for 117 days. Edemas disappeared and no displacement of pelvic organs was observed by CAT-scan monitoring. Objectively measured partial responses were observed in 3 patients. A 52-year-old male having a local relapse of a larynx carcinoma with invasion of lymphatic nodes had tumors on the right and left sides of the neck. Thirty-day treatment at 0.12 mg/m² showed a reduced lymphatic nodes mass in partial response to such treatment. This patient could not be followed additionally because of a severe intercurrent pulmonary infection. A 62-year-old man with nondifferentiated thyroid carcinoma and metastases in the right axillary

nodes, at a dose of 0.21 mg/m² crotoxin, showed a partial response on study day 90, which remained stable for another 30-day cycle. A 50-year-old woman having a rectal carcinoma and pelvic metastases, with a measurable nonresectable tumor in the upper vaginal area, received a dose of 0.21 mg/m². A CAT-scan on day 78 showed reduced dimensions, which allowed surgical removal of the tumor. Finally, a patient with mammary carcinoma and metastasis in the right lung, pleural effusion, and multiple bone lesions received 0.21 mg/m² crotoxin. CAT-scan studies on day 90 showed a complete response in both lung and bone lesions, which lasted for 6 months after suspension of treatment.

Previous study treatment of responding patients is shown in Table 1* .

► DISCUSSION

The safety profile of crotoxin administered i.m. to humans showed neurotoxicity as the most conspicuous toxic effect. This is an expected toxicity, consistent with the known effects of this toxin (31, 32, 33) . Low crotoxin concentrations produce incomplete blockage (34) reflected *in vivo* by paresis, which is fully reversible within 24–40 h (29) . The neurotoxic activity of crotoxin may account for diplopia, which appeared in all of the patients at the dose level of 0.18 mg/m² because of paresis of the external ocular muscles. Diplopia was regarded as nonlimiting toxicity, because it disappeared at least 12 h before the next dose and progressively lasted for shorter intervals of time. Diplopia was not, usually during the second week, spontaneously reported by the patients, and eventually it disappeared completely. At the MTD, paresis was self-limited and did not affect the intrinsic ocular muscles or extend to pharyngeal, laryngeal, or respiratory muscles. Furthermore, it was spontaneously reversible and did not require any dose adjustment. However, for Phase II studies it may be advisable to exclude patients with primary or paraneoplastic myasthenia.

At the dose level of 0.22 mg/m² diplopia was accompanied at times by strabismus and palpebral ptosis indicating increased neuromuscular impairment. Two of 6 patients had incoordination and nystagmus accompanied by grade 2–3 anxiety. Ataxia and incoordination were reported in previous studies (23) and in preclinical studies in dogs (30) . Nystagmus was reported in cats (23) . All are signs of severe crotoxin toxicity. Therefore, they were specifically regarded as DLT in this study. Because nystagmus episodes were accompanied by grade 2–3 anxiety, no additional dose escalation was considered thereafter. Additionally, because the difference between MTD and DLT is surprisingly small, the recommended Phase II dose should be set at 0.18 mg/m².

Other toxic effects such as sialorrhea (23 , 30) , miction, and diarrhea (30) are cholinergic symptoms, which have been reported in preclinical studies in dogs. Also, when injected i.m., crotoxin has myotoxic activity (27 , 28) . Preclinical toxicity studies in dogs found an increase in plasma levels of CK, ALT, AST, and lactic dehydrogenase within 2 h, reaching a peak at 18–40 h after injection and returning to normal values on days 3–4 without additional pathological or biochemical evidences of liver damage (30) . Clinical, pathological, and biochemical data in preclinical studies consistently showed

that crotoxin-induced signs of toxicity decreased or disappeared on continuation of the treatment (29, 30). This appears to be related to induction of tolerance to crotoxin-induced neuro- and myotoxic effects. Crotoxin is an heterologous protein, and a significant adverse effect may be hypersensitive reaction. Six patients presented indirect evidences of sensitization such as eosinophilia, and almost all of the patients receiving 0.21 mg/m² crotoxin had specific anticrotoxin antibodies after week 4 of treatment. Only 1 patient showed an anaphylactic reaction (grade 3) on study day 31 and had to be separated from the study.

At the doses used, crotoxin administration did not affect hematopoietic, hepatic, or renal functions. This coincides with results from preclinical studies (30). Finally, 18 of the 23 evaluable patients reported a decrease or disappearance of pain. This effect was unexpected and at times remarkable. A 25-year old male with Ewing's sarcoma increased mobility after diminishing of pain and gained muscular strength. A 46-year-old male patient with a rectal carcinoma and pelvic invasion suspended the regularly administered morphine after 3 weeks of treatment on 0.12 mg/m² crotoxin. This effect persisted with no daily fluctuations, even when disease progression was detected. At present, there is no clear explanation for this effect, which deserves a separate investigation.

Pharmacokinetic data fitted to a two-compartment open model with absorption after a first order kinetics. After i.m. injection, absorption from the injection site and distribution from the central to the peripheral compartment were both rapid and almost complete within 2 h after injection (*i.e.*, the interval of time after injection at which manifestation of crotoxin-related side effects occur). The half-life of the elimination phase was 5.2 ± 0.6 h, indicating that in 24 h ($\sim 4.8 \pm 0.12$ half-lives) $\sim 95\%$ of the toxin has been eliminated. This is in agreement with studies on distribution of ¹²⁵I-crotoxin in mice (9). This result is also consistent with the lack of cumulative toxicity found in mice (29) and dogs (30) receiving daily doses of crotoxin.

Previous studies showed that crotoxin does not bind to erythrocyte membranes (13, 14) or to plasma proteins (9). Therefore, the fraction of crotoxin unbound in plasma $f_U = 1$. In the extravascular fluid crotoxin binds to specific acceptor sites in neural and muscular tissues (13, 14) without being internalized (34). Thus, the fraction of unbound crotoxin to tissues $f_{UR} \ll 1$. This is consistent with an apparent volume of distribution (12 liter/kg) much larger than the sum of the volume of plasma ($V_p \sim 43$ ml/kg⁻¹) plus the volume of extravascular fluid ($V_e \sim 170$ ml/kg⁻¹; Ref. 35). At 720 min after injection, the absorption and distribution phases have been completed. Using the pharmacokinetic equation of Fig. 1* the amount of crotoxin remaining is ~ 910 ng/kg, and the plasma concentration is 0.075 ng/ml⁻¹.

Thus, plasma will contain (42 ml/kg x 0.075 ng/ml) 3.14 ng of crotoxin. In the extravascular fluid, the amount of free crotoxin will be (170 ml/kg x 0.075 ng/ml) 10.45 ng/kg, and the remaining 895.4 ng/kg will be bound crotoxin, so that $f_{UR} = 10.45/895.4 = 0.0116$. Therefore the $f_U/f_{UR} = 86.6$. The apparent volume of distribution will be (35):

$$V_d = V_p + (V_{ev} \times f_U/f_{UR}) = 42 + (170 \times 86.6) = 12,166 \text{ ml/kg (or 12.16 liter/kg).}$$

On study day 15, the AUC is unchanged and C_L will be similar, whereas the calculated V_d is reduced. These changes coincide with the onset of tolerance to crotoxin toxicity

and may reflect an increase in f_{UR} because of either the decrease in the density of acceptor sites or a reduced affinity for the toxin, as suggested in preclinical studies (29).


After study day 30, the changes in plasma concentration *versus* time curves can relate to the presence of anticrotoxin antibodies in patient plasma. Formation of anticrotoxin antibodies is expected as the result of the immune response to daily administration of a heterologous protein. Thus, a fraction of the crotoxin administered will be rapidly trapped as immune complexes by antibodies, lowering the increase in plasma concentration resulting from absorption. This will be reflected in the plasma concentration *versus* time curve as a decreased absorption rate. Because a larger amount of toxin has to be absorbed to reach a concentration level at which the rate of absorption equals the rate of elimination (which is unchanged) the peak concentration would occur later and be lower. Consequently, the AUC will be reduced. The presence of anticrotoxin antibodies has clinical implications, because they may neutralize the effects of the toxin, and their concentration increase with time. For example the plasma from a patient treated with crotoxin at the dose level of 0.21 mg/m^2 on study day 31 neutralized $30 \pm 10 \text{ ng/ml}$ crotoxin, and the AUC was $\sim 140 \text{ ng/min/ml}$. On study day 90 it neutralized $60 \pm 20 \text{ ng/ml}$ crotoxin, and the AUC was about 95 ng/min/ml . One approach to circumvent this problem may be based on the consideration that anticrotoxin antibodies just decrease the availability (F) of crotoxin. Because $F \times (\text{dose}) = \text{AUC} \times C_L$, if the clearance is constant, the ratio $(\text{AUC})_{\text{DAY 1}} : (\text{AUC})_{\text{DAY 30}}$ for each patient will give the factor by which the crotoxin dose is to be increased on study day 30. However, as the crotoxin dose is adjusted, it may produce much higher levels of anticrotoxin antibodies after study day 30. Another approach could consist on pretreatment with inactive crotoxin. Because PLA_2 activity is needed for biological activity, and this enzyme can be inactivated (4), the modified crotoxin will be fully recognized by anticrotoxin antibodies, although its enzymatic activity as well as toxicity are irreversibly lost. This procedure may be used to lower the antibody titers, provided that it does not give rise to anaphylactic reactions.


In summary, crotoxin is a new class of anticancer agent acting through a novel mechanism of action. Neurotoxicity is the principal toxic effect and appears to be manageable, as shown under the present study. The therapeutic response obtained in some patients is quite promising and deserves additional development of this compound under a Phase II clinical trial. The recommended dose for Phase II studies is 0.18 mg/m^2 .

► FOOTNOTES

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³ The abbreviations used are: PLA₂, phospholipase A₂; MTD, maximum tolerated dose; CK, creatinine kinase; AST, asparagine transaminase; ALT, alanine transaminase; CAT, computer-assisted tomography; ECOG, Eastern Cooperative Oncology Group; DLT, dose-limiting toxicity; LD₅₀, lethal dose 50%; CI, confidence interval; FVC, forced vital capacity; AUC, area under curve. 

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