

# Phase I and Pharmacokinetics Study of Crotoxin (Cytotoxic PLA<sub>2</sub>, NSC-624244) in Patients with Advanced Cancer<sup>1</sup>

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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/m<sup>2</sup>. 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/m<sup>2</sup> 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/m<sup>2</sup>. 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/m<sup>2</sup> 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/m<sup>2</sup> and 0.22 mg/m<sup>2</sup>) 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 eosino-

philia during the first 2 weeks. The maximum tolerated dose was set at 0.21 mg/m<sup>2</sup>. Objective measurable partial responses (>50% reduction of tumor mass) were noted in 2 patients treated at 0.21 mg/m<sup>2</sup> and 1 at 0.12 mg/m<sup>2</sup>. One patient (at 0.21 mg/m<sup>2</sup>) presented a complete response on day 110. Crotoxin pharmacokinetics showed rapid absorption from the injection site to blood ( $t_{1/2A} = 5.2 \pm 0.6$  min). Plasma concentration reached a peak ( $C_{max} = 0.79 \pm 0.1$  ng/ml) at  $\tau_{max} = 19 \pm 3$  min. The half-life of the distribution ( $\alpha$ ) phase is  $22 \pm 2$  min. Starting at 1.5 h after injection, the decrease in plasma concentration becomes slower, reaching  $14 \pm 3$  pg/ml 24 h after injection. The profile is dominated by the elimination ( $\beta$ ) phase with a half-life of  $5.2 \pm 0.6$  h. Consequently, 24 h after the injection ( $\sim 5$  half-life) 97% of the product was eliminated. The area under plasma concentration versus time curve was  $0.19 \pm 0.05$   $\mu\text{g}/\text{min}/\text{ml}$ . Assuming availability (F)  $\sim 1$ , 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/m<sup>2</sup>.

## INTRODUCTION

Crotoxin is a cytotoxic PLA<sub>2</sub><sup>3</sup> 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  $\sim 9.5$  kDa) and one basic (subunit B  $\sim 14.5$  kDa). Subunit B is a PLA<sub>2</sub> formed by a single chain of 122 amino acid residues cross-linked by seven disulfide bonds (1-6). 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 (LD<sub>50</sub> i.v. mice  $> 20$  mg/kg; Ref. 6). The two subunits form spontaneously a tight 1:1 complex (6, 8). Complex formation inhibits the PLA<sub>2</sub> activity but increases toxicity by at least one order of magnitude (6, 9-11). 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 <sup>125</sup>I-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 PLA<sub>2</sub> from an unspecific cytotoxin into a self-target toxin compound.

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<sup>3</sup> The abbreviations used are: PLA<sub>2</sub>, phospholipase A<sub>2</sub>; MTD, maximum tolerated dose; CK, creatinine kinase; AST, aspartate aminotransferase; ALT, alanine transaminase; CAT, computer-assisted tomography; ECOG, Eastern Cooperative Oncology Group; DLT, dose-limiting toxicity; LD<sub>50</sub>, lethal dose 50%; CI, confidence interval; FVC, forced vital capacity; AUC, area under curve.

Crotoxin displays cytotoxic activity against a variety of murine (4) and human tumor cell lines *in vitro* (18), which require both the PLA<sub>2</sub> 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 PLA<sub>2</sub> (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 (23–25). 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 IC<sub>50</sub> values, and, in accordance with previous studies on organ distribution of <sup>125</sup>I-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

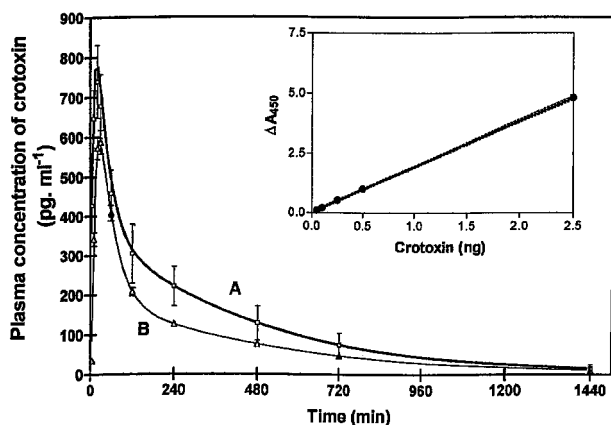
**Eligibility.** Patients entered had histologically confirmed solid tumors not treatable by surgery, radiation therapy, or standard chemotherapy. Eligibility criteria included the following:

(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 ≥1,500/mm<sup>3</sup>; platelet count ≥ 150,000/mm<sup>3</sup>; hemoglobin level ≥ 9 g/dl), renal (serum creatinine ≤ 2 mg/dl), and liver function (AST and ALT ≤ 3 × 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 ≥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<sup>2</sup> (*i.e.*, 1/20 of the LD<sub>10</sub> 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<sup>2</sup>, 0.12 mg/m<sup>2</sup>, and 0.18 mg/m<sup>2</sup>. Additional increases in dose levels were smaller: 0.21 mg/m<sup>2</sup> and 0.22 mg/m<sup>2</sup>. 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 × 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.



**Fig. 1** Plasma concentration versus time curves of crotoxin after the i.m. administration to patients at the dose level of 0.21 mg/m<sup>2</sup>. 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 ng/ml, and the exponential coefficients represent the rate constants for the distribution, elimination, and absorption phases, in that order, expressed in min<sup>-1</sup>. Curve B, data obtained for a patient after the i.m. injection of crotoxin at the dose level of 0.21 mg/m<sup>2</sup> on study day 30. Inset, calibration curve for determination of crotoxin. The change in absorbance ( $\Delta A_{450}/\text{min}/\text{ml} \times$  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 =  $\pm 0.013$ ; intercept =  $0.0318 \pm 0.0153$ ; and  $r^2 = 0.998$ . Dotted lines, the limits of 95% CI.

**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<sub>2</sub>-terminal analysis of both subunits were consistent with data published previously. The isolated subunit B had a PLA<sub>2</sub>-specific activity of  $76 \pm 6$  units/mg protein (pH-Stat) and an LD<sub>50</sub> (i.v., mice) of  $0.56 \pm 0.12$   $\mu\text{g}/\text{g}$ . No enzymatic activity or toxicity was observed associated with subunit A. The reconstituted crotoxin complex gave a single peak ( $24 \pm 0.2$  kDa) by analytical gel filtration on a prepacked Superdex 75 HR column, and had a PLA<sub>2</sub>-specific activity of  $21 \pm 3$  units/mg protein and an LD<sub>50</sub> (i.v., mice) of  $0.06 \pm 0.015$   $\mu\text{g}/\text{g}$ . Analysis by SDS-PAGE (PhastGel 8–25% gradient) under nonreducing conditions (silver stain) showed two bands with molecular weights  $9.5 \pm 0.3$  kDa (subunit A) and  $14.5 \pm 0.2$  kDa (subunit B). The LD<sub>10</sub> i.m. in mice was estimated at 0.19  $\mu\text{g}/\text{g}$  (95% CI, 0.13–0.23). Crotoxin samples were dissolved in sterile, pyrogen-free PBS, sterilized by filtration, diluted to a final concentration of  $2.0 \pm 0.08$  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,

**Table 1** Patients characteristics<sup>a</sup>

A. Enrollment data			
Entered (total)	26		
Entered and evaluated	23		
Median age (range) (yr)	52 (25–76)		
Sex (M/F)	12/11		
ECOG Performance Status			
0	3		
1	14		
2	6		
B. Tumor description and metastasis			
Tumor type	No. of patients	No. of metastasis	No. of patients
Bladder	1		
Breast	4	0	0
Cervix	2	1	5
Gastrointestinal	7	2	6
Larynx	1	$\geq 3$	15
Lung (non-small cell lung cancer)	3		
Prostate	2		
Head and neck	1		
Fallopian adenocarcinoma	1		
Fibrosarcoma	1		
Ewing's sarcoma	1		
Thyroid carcinoma	1		
Liposarcoma	1		
C. Prior treatment			
Chemotherapy	25 (1 cycle 5 pts, 2 cycles 12 pts, 3 cycles 8 pts)		
Mytomyacin C/nitrosurea	2		
Immunotherapy	5		
Other (antihormone therapy)	4		
Radiation therapy	20		
D. Responding patients prior treatment			
Principal diagnosis	Prior study treatment		
Gastroduodenal + hepatomegalia	Surgery and chemotherapy (2 cycles)		
Thyroid carcinoma	Surgery, chemotherapy (2 cycles), radiation		
Fibrosarcoma	Chemotherapy (1 cycle), immunotherapy		
Larynx carcinoma	Surgery, chemotherapy (1 cycle), radiation		
Rectal carcinoma	Chemotherapy (3 cycles)		
Breast	Surgery, chemotherapy (2 cycles), radiation, and hormone therapy		

<sup>a</sup> One patient with fibrosarcoma died on study day 3 for reasons unrelated to drug treatment. Another patient with gastrointestinal cancer presented a biliary obstruction and was removed from protocol on study day 6 for treatment. A patient with prostate cancer abandoned the protocol on study day 8, unable to adapt to the obligatory in-patient protocol conditions.

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

Table 2 Neurotoxicity in patients treated with crotoxin

Dose (mg/m <sup>2</sup> )	Days of treatment	Neurotoxicity <sup>a</sup> grade (study days)			
		Diplopia <sup>b</sup>	Palpebral ptosis	Nystagmus	Anxiety
0.18	30	2 (9–19)	—	—	—
0.18	30	2 (6–14)	—	—	—
0.18	30	2 (2–10)	—	—	—
0.21	30	2 (2–20)	—	—	—
0.21	30	2 (1–14)	—	—	—
0.21	90	2 (11–21)	—	—	—
0.22	30	2 (2–4)	(19–22)	2 (15–16)	2 (15), 3 (16)
0.22	90	2 (6–14)	—	2 (16–17)	3 (17)
0.22	30	2 (8)	—	—	—
0.22	30	2 (4, 5)	—	—	—
0.22	30	2 (2–21)	(18–20)	2 (15)	3 (15)
0.22	30	2 (8–21)	(12–18)	2 (19–20)	2 (19); 3 (20)
0.21	2	—	Died on study day 3 <sup>c</sup>		
0.21	90	2 (2–21)	—	—	—
0.21	60	2 (2–11)	—	—	—
0.21	90	2 (1–5)	—	—	—
0.21	30	2 (10–21)	Discharged on study day 31 <sup>d</sup>		
0.21	8	2 (4–5)	Abandoned protocol on day 8 <sup>e</sup>		

<sup>a</sup> No neural toxicity was observed at doses up to 0.18 mg/m<sup>2</sup>.

<sup>b</sup> Diplopia appeared intermittently within the study days indicated in parenthesis.

<sup>c</sup> This patient died on study day 3 of pulmonary edema.

<sup>d</sup> Patient had a mild allergic reaction on study day 3 and a grade 3 anaphylactic reaction on day 31.

<sup>e</sup> Patient abandoned the protocol on study day 8, unable to adapt to the obligatory in-patient condition required by this study.

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<sup>2</sup> 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 × 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 µg of immunopurified anticrotoxin horse IgG. This method was used to determine crotoxin con-

centration 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 0.05–2.5 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<sub>450</sub> nm after the addition of 5.5 mM o-phenylene diamine (Sigma Chemical Co.) and 3 mM hydrogen peroxide. “Blank” eluates gave ΔA<sub>450</sub> values of <0.001/min. Five or six samples with different volumes of each eluate were measured to ensure linearity between volume and ΔA<sub>450</sub>/min. The minimum acceptable values of ΔA<sub>450</sub> was 0.015/min. As shown in Fig. 1 (inset) the plot of total ΔA<sub>450</sub> [i.e., ΔA<sub>450</sub>/min (± SD)] × total volume of eluate is a straight line. R<sup>2</sup> = 0.999 with a slope 1.913 (95% CI, 1.874–1.973) ΔA<sub>450</sub>/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

Table 3 Non-neurological toxicity

Dose (mg/m <sup>2</sup> )	Clinical signs		Laboratory			Remarks
	FVC	Other	Eosinophilia	ALT <sup>a</sup>	AST <sup>a</sup>	
					Grade	
0.06	—	—	—	—	—	Discharged <sup>b</sup>
0.06	—	—	+	—	—	DP <sup>c</sup>
0.12	—	—	—	1	2	DP
0.12	—	Sialorrhea, IBP <sup>d</sup>	—	1	2	DP
0.18	↓	—	—	2	2	DP
0.18	—	Diarrhea, IBP	—	2	2	SD <sup>e</sup> A.T. <sup>f</sup>
0.21	↕	—	—	2	2	CR <sup>g</sup>
0.22	—	—	+	2	2	SD A.T. <sup>f</sup>
0.22	—	—	+	2	2	PR <sup>h</sup>
0.22	↓	Sialorrhea	+	2	2	DP
0.21	—	—	—	2	2	PR
0.21	↓	—	+	2	2	DP
0.21	—	—	+	1	2	PR
0.21	—	Diarrhea, IBP, Anaphylaxis	—	2	2	Discharged <sup>b</sup>
0.21	—	IBP	—	1	2	A.T. <sup>h</sup>

<sup>a</sup> The increases in ALT, AST, and CK listed are observed during the first 2 weeks of treatment. They decrease during the third week and return to normal during the fourth week of treatment.

<sup>b</sup> Patient was removed from protocol on study day 6 because of biliary obstruction and required surgery. Another patient presented grade 3 anaphylaxis on study day 31.

<sup>c</sup> DP, disease progression.

<sup>d</sup> Grade 1 increase in blood pressure.

<sup>e</sup> SD, stable disease. Three patients abandoned the treatment after the first 30-day cycle, unable to continue with the obligatory in-patient condition required for this study.

<sup>f</sup> A.T., abandoned treatment.

<sup>g</sup> CR, complete response.

<sup>h</sup> PR, measurable partial response.

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  $-20^{\circ}\text{C}$  until assay. Crotoxin concentration was assayed by “double sandwich” ELISA on plates sensitized with 2  $\mu\text{g}$  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  $\mu\text{l}$ . Incubations were performed for 60 min at  $30^{\circ}\text{C}$  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.

**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

$C_{\text{max}}$  and  $\tau_{\text{max}}$  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.

**Neurological Toxicity.** No significant toxicity was observed up to the dose level of 0.12 mg/m<sup>2</sup>. At dose levels 0.18

Table 4 Pharmacokinetic parameters of crotoxin administered i.m. to patients at the dose of 0.21 mg/m<sup>2</sup> on study days 1 and 15

Parameter (units)	Day 1 × (± SD)	Day 15 × (± SD)
<sup>a</sup> C (ng/ml <sup>-1</sup> )	1.53 (±0.14)	1.63 (±0.12)
<sup>a</sup> K <sub>A</sub> (min <sup>-1</sup> )	0.121 (±0.003)	0.173 (±0.002)
<sup>a</sup> t <sub>1/2A</sub> (min)	5.2 (±0.6)	4.8 (±0.3)
<sup>b</sup> A (ng/ml <sup>-1</sup> )	1.11 (±0.16)	1.2 (±0.2)
<sup>b</sup> α (min <sup>-1</sup> )	0.032 (±0.01)	0.038 (±0.02)
<sup>b</sup> t <sub>1/2α</sub> (min)	22 (±3)	18.2 (±4.2)
<sup>c</sup> B (ng/ml <sup>-1</sup> )	0.38 (±0.12)	0.38 (±0.10)
<sup>c</sup> β (h <sup>-1</sup> )	0.136 (±0.03)	0.163 (±0.03)
<sup>c</sup> t <sub>1/2β</sub> (h)	5.2 (±0.6)	4.3 (±0.6)
<sup>d</sup> C <sub>L</sub> (ml/min <sup>-1</sup> )	26.0 (±8)	25.5 (±8)
AUC (μg·min/ml <sup>-1</sup> )	0.190 (±0.05)	0.196 (±0.03)
<sup>e</sup> V <sub>d</sub> (liter/kg <sup>-1</sup> )	12 (±3)	9.1 (±4)

<sup>a</sup> C, K<sub>A</sub>, and t<sub>1/2A</sub> are the coefficient, rate constant, and half-life for the absorption phase, respectively.

<sup>b</sup> A, α, and t<sub>1/2α</sub> are the coefficient, rate constant, and half-life for the distribution phase, respectively.

<sup>c</sup> B, β, and t<sub>1/2β</sub> are the coefficient, rate constant, and half-life for the elimination phase, respectively.

<sup>d</sup> C<sub>L</sub> = Clearance.

<sup>e</sup> V<sub>d</sub>, apparent volume of distribution.

and 0.21 mg/m<sup>2</sup> 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/m<sup>2</sup> 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/m<sup>2</sup>. 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.

**Nonneurological Toxicities.** Nonneurological toxicities are presented in Table 3.

A 58-year-old female with cervix cancer treated at dose level of 0.21 mg/m<sup>2</sup> 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/m<sup>2</sup>, and a 58-year-old male with lung cancer, treated at 0.22 mg/m<sup>2</sup>) 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/m<sup>2</sup> 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/m<sup>2</sup> on study days 1 and 30 is shown in Fig. 1. The toxin was rapidly absorbed from the site of injection (t<sub>1/2A</sub> = 5.2 ± 0.6 min) to the blood. Crotoxin concentration in plasma reached a peak (C<sub>max</sub> = 770 ± 110 pg/ml) at τ<sub>max</sub> = 19 ± 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 (C<sub>L</sub>) is 25 ± 8 ml/min, the AUC is 0.19 ± 0.05 μg·min/ml, and the apparent volume of distribution (V<sub>d</sub>) is ~12 ± 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 ± 2% of the dose. Renal clearance (CL<sub>R</sub>) 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.

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<sub>max</sub> = 0.95 ± 0.12 ng/ml) seems to be higher and appears sooner (τ<sub>max</sub> = 15 ± 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<sub>d</sub>) = 9.1 ± 4 liters/kg.

Changes in plasma concentration *versus* time curves on

Table 5 Urine data following the i.m. injection of crotoxin (0.21 mg/m<sup>2</sup>)

Time of collection (h)	Volume of urine (ml)	Crotoxin in urine (ng/ml)	Amount excreted in time interval (μg)	Excretion rate (μg/h)	AUC within interval (ng/min/ml)
0–2	100 ± 20	68.5 ± 7.5	7 ± 2	3.5 ± 1	61 ± 10
2–4	160 ± 32	21.2 ± 5.0	3.5 ± 1.4	1.75 ± 1.9	35 ± 7
4–6	90 ± 22	25.1 ± 5.2	2.8 ± 1.3	1.4 ± 0.6	28 ± 9
6–8	340 ± 46	5.9 ± 1.9	2.1 ± 0.9	1.05 ± 0.4	16 ± 4
8–12	192 ± 18	18.3 ± 3.1	3.5 ± 1.0	0.88 ± 0.5	23 ± 6
12–24	910 ± 43	3.1 ± 2.0	2.8 ± 2.4	0.23 ± 0.2	20 ± 3

study day 30 (Fig. 1) consisting in an apparent decrease in the absorption rate ( $K_A = 0.07 \pm 0.02 \text{ min}^{-1}$ ) showed a lower peak of plasma concentration ( $C_{\text{max}} = 0.6 \pm 0.3 \text{ ng/ml}$ ) appearing later ( $\tau_{\text{max}} = 23 \pm 3 \text{ min}$ ) and with a smaller AUC ( $0.14 \pm 0.07 \mu\text{g}/\text{min}/\text{ml}$ ), 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  $0.06 \text{ mg}/\text{m}^2$  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  $0.21 \text{ mg}/\text{m}^2$  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  $0.12 \text{ mg}/\text{m}^2$  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  $0.21 \text{ mg}/\text{m}^2$  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 non-resectable tumor in the upper vaginal area, received a dose of  $0.21 \text{ mg}/\text{m}^2$ . 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  $0.21 \text{ mg}/\text{m}^2$  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 (31–33). 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  $0.18 \text{ mg}/\text{m}^2$  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  $0.22 \text{ mg}/\text{m}^2$  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  $0.18 \text{ mg}/\text{m}^2$ .

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. Cro-

toxin 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/m<sup>2</sup> crotoxin had specific anti-crotoxin 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/m<sup>2</sup> 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 (~4.8 ± 0.12 half-lives) ~95% of the toxin has been eliminated. This is in agreement with studies on distribution of <sup>125</sup>I-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<sup>-1</sup>) plus the volume of extravascular fluid ( $V_e \sim 170$  ml/kg<sup>-1</sup>; 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<sup>-1</sup>.

Thus, plasma will contain (42 ml/kg × 0.075 ng/ml) 3.14 ng of crotoxin. In the extravascular fluid, the amount of free crotoxin will be (170 ml/kg × 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 an 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<sup>2</sup> on study day 31 neutralized 30 ± 10 ng/ml crotoxin, and the AUC was ~140 ng/min/ml. On study day 90 it neutralized 60 ± 20 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<sub>2</sub> 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<sup>2</sup>.

## REFERENCES

- Aird, S. D., Kaiser, I. I., Lewis, R. V., and Kruggel, W. G. A complete amino acid sequence for the basic subunit of crotoxin. *Arch. Biochem. Biophys.*, 249: 296–300, 1986.
- Bon, C., Changeux, J. P., Jeng, T. W., Fraenkel-Conrat, H. Post-synaptic effects of crotoxin and its isolated subunits. *Eur. J. Biochem.*, 99: 471–481, 1979.
- Faure, G., Harvey, A. L., Thommson, E., Saliou, B., Radvanyi, F., and Bon, C. Comparison of crotoxin isoforms reveals that stability of the complex plays a major role in its pharmacological action. *Eur. J. Biochem.*, 214: 491–496, 1993.
- Corin, R. E., Viskatis, L. J., Vidal, J. C., and Etcheverry, M. A. Cytotoxicity of crotoxin on murine erythroleukemia cells *in vitro*. *Investig. New Drugs*, 11: 11–15, 1993.



5. Kini, M. R., and Evans, H. J. A common cytolytic region in myotoxins, hemolysins, cardiotoxins and antibacterial peptides. *Int. J. Peptide Protein Res.*, *34*: 277–286, 1989.
6. Habermann, E., and Breithaupt, H. Mini-Review. The crotoxin complex—an example of biochemical and pharmacological protein complexation. *Toxicon*, *16*: 19–30, 1978.
7. Aird, S. D., Kaiser, I. I., Lewis, R. V., and Kruggel, W. A. Rattlesnake presynaptic neurotoxins. Primary structure and evolutionary origin of the acidic subunit. *Biochemistry*, *24*: 7050–7058, 1985.
8. Radvanyi, F., and Bon, C. Catalytic activity and reactivity with p-bromophenacyl bromide of the phospholipase subunit of crotoxin. *J. Biol. Chem.*, *257*: 12616–12623, 1982.
9. Habermann, E., Walsch, P., and Breithaupt, H. Biochemistry and pharmacology of the crotoxin complex II. Possible interrelationships between toxicity and organ distribution phospholipase A2, crotopotin and their combination. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, *273*: 313–330, 1972.
10. Hawgood, B. J., and Smith, J. W. The mode of action at the mouse neuromuscular junction of the phospholipase A-crotopotin complex isolated from the venom of the South American Rattlesnake. *Br. J. Pharmacol.*, *61*: 597–606, 1977.
11. Hawgood, B. J., and Santana de Sa, S. Changes in spontaneous and evoked release of transmitter induced by the crotoxin complex and its component phospholipase A2 at the frog neuromuscular junction. *Neuroscience*, *4*: 293–303, 1979.
12. Chang, C., and Su, M. J. A study of the interaction of crotopotin with crotoxin phospholipase A2, notexin and other presynaptic neurotoxins. *Br. J. Pharmacol.*, *73*: 495–503, 1981.
13. Degn, L. L., Seebart, C. S., and Kaiser, I. I. Specific binding of crotoxin to brain synaptosomes and synaptosomal membranes. *Toxicon*, *29*: 973–988, 1991.
14. Delot, E., and Bon, C. Model for the interaction of crotoxin, a phospholipase A2 neurotoxin with presynaptic membranes. *Biochemistry*, *32*: 10708–10713, 1993.
15. Lambeau, G., Barhanin, J., Schweitz, H., Qar, J., and Lazdunski, M. Identification and properties of very high affinity brain membrane-binding sites for a neurotoxic phospholipase from the taipan venom. *J. Biol. Chem.*, *264*: 11503–11510, 1989.
16. Lambeau, G., Schmid-Alliana, A., Lazdunski, M., and Barhanin, J. Identification and purification of a very high affinity binding protein for toxic phospholipases A2 in skeletal muscle. *J. Biol. Chem.*, *265*: 9526–9532, 1990.
17. Radvanyi, F., Saliou, B., Lambezat, M. P., and Bon, C. Binding of crotoxin, a phospholipase A2 neurotoxin to negatively charged phospholipid vesicles. *J. Neurochem.*, *53*: 1252–1260, 1989.
18. Rudd, C. L., Viskatis, L. J., Vidal, J. C., and Etcheverry, M. A. *In vitro* comparison of cytotoxic effects of crotoxin in three human tumor cell lines and a normal human keratinocyte cell line. *Investig. New Drugs*, *12*: 183–184, 1994.
19. Donato, N. J., Martin, C. A., Perez, M., Newman, R. A., Vidal, J. C., and Etcheverry, M. A. Regulation of epidermal growth factor receptor activity by crotoxin, a snake venom phospholipase A2 toxin. *Biochem. Pharmacol.*, *51*: 1535–1543, 1996.
20. Fletcher, J. E., Selistre de Araujo, H. S., and Ownby, C. L. Molecular events in the myotoxic effects of phospholipases. *In*: R. M. Kini (ed.). *Venom Phospholipase A2 Enzymes. Structure, Function and Mechanism*, pp. 455–497. New York: John Wiley & Sons, 1997.
21. Ownby, C. L., Selistre de Araujo, H. S., White, S. P., and Fletcher, J. E. Lysine-49 phospholipase A2 proteins. *Toxicon*, *37*: 411–445, 1999.
22. Newman, R. A., Vidal, J. C., Viskatis, L. J., Johnson, J. I., and Etcheverry, M. A. A Novel compound of purified animal toxins separates antitumor efficacy from Neurotoxicity. *Investig. New Drugs*, *11*: 151–159, 1993.
23. Vital-Brazil, O., Prado-Franceschi, J., and Waisbich, E. Pharmacology of Crystalline crotoxin I. *Toxic. Mem. Inst. Butantan*, *33*: 973–980, 1966.
24. Breithaupt, H. Neurotoxic and myotoxic effects of *Crotalus* phospholipase A2 and its complex with crotopotin. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, *292*: 271–298, 1976.
25. Chang, C. C., and Lee, J. D. Crotoxin, the neurotoxin of South American rattlesnake venom, is a presynaptic toxin actin like  $\beta$ -bungarotoxin. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, *296*: 159–168, 1977.
26. Gopalakrishnakone, P., Dempster, D. W., Hawgood, B. J., and Elder, H. Y. Cellular and mitochondrial changes induced in the structure of murine skeletal muscle by crotoxin, a neurotoxic phospholipase A2 complex. *Toxicon*, *22*: 85–98, 1984.
27. Mebs, D., and Ownby, C. L. Myotoxic components of snake venoms: their biochemical and biological activities. *Pharmacol. Ther.*, *48*: 223–236, 1990.
28. Kouyoumedjian, J. A., Harris, J. B., and Johnson, M. A. Muscle necrosis caused by the subunits of crotoxin. *Toxicon*, *24*: 575–583, 1986.
29. Okamoto, M., Viskatis, L. J., De la Roza, G., and Vidal, J. C. Induction of tolerance to crotoxin in mice. *J. Pharmacol. Exp. Ther.*, *265*: 41–46, 1993.
30. De Tolla, L. J., Stump, K. C., Russell, R., Viskatis, L. J., Vidal, J. C., Newman, R. A., and Etcheverry, M. A. Toxicity of the novel animal-derived anticancer agent, VRCTC-310: acute and subchronic studies in beagle dogs. *Toxicology*, *99*: 31–46, 1995.
31. Vital-Brazil, O., and Excell, B. J. Action of crotoxin and crotactin from the venom of *Crotalus durissus terrificus* (South American rattlesnake) on the frog neuromuscular Junction. *J. Physiol. (Lond.)*, *212*: 34P–35P, 1970.
32. Lee, C. Y., and Ho, C. L. The pharmacology of phospholipases A2 isolated from snake venoms, with particular reference to their effects on neuromuscular transmission. *In*: H. Yoshida, Y. Hagihara, and E. Ebashi (eds.). *Advances in Pharmacology and Therapeutics*, Vol. 4, pp. 37–52. Oxford, United Kingdom: Pergamon Press, 1982.
33. Strong, P. N. Presynaptic phospholipase A2 neurotoxins: Relationship between Biochemical and electrophysiological approaches to the mechanism of toxin action. *In*: (M. J. Dowdall and J. N. Hawthorne, eds.). *The Cellular and Molecular Basis of Cholinergic Function*, pp. 534–549. Chichester: Ellis Horwood, 1987.
34. Trivedi, S., Kaiser, I. I., Tanaka, M., and Simpson, L. L. Pharmacologic experiments on the interaction between crotoxin and the mammalian neuromuscular junction. *J. Pharmacol. Exp. Ther.*, *251*: 490–496, 1989.
35. Rowland, M., and Tozer, T. N. *Clinical Pharmacokinetics*, Ed. 2, pp. 33–48 and pp. 438–450. Philadelphia: Lea & Freiberg, 1989.

# Clinical Cancer Research

## Phase I and Pharmacokinetics Study of Crotoxin (Cytotoxic PLA<sub>2</sub>, NSC-624244) in Patients with Advanced Cancer

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