



DDI Toolbox Information Submission Form

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B: Tool Type Submitted

Tool Type
<input type="checkbox"/> In vitro model
<input type="checkbox"/> In vivo model
<input checked="" type="checkbox"/> Candidate therapeutic
<input type="checkbox"/> Drug delivery technology
<input type="checkbox"/> Other _____

C: Tool Type: Details

Check as many as apply

Relevant to Disorder	Screening Models	Therapeutic Focus within NF	Signaling pathway/target
<input checked="" type="checkbox"/> NF1	In vitro models:	<input checked="" type="checkbox"/> Plexiform neurofibroma	<input checked="" type="checkbox"/> Growth factor receptor modulator
<input checked="" type="checkbox"/> NF2	<input type="checkbox"/> Cell line (human)	<input checked="" type="checkbox"/> Neurocutaneous fibroma	<input type="checkbox"/> Ras-dependent
<input checked="" type="checkbox"/> Schwannomatosis	<input type="checkbox"/> Cell line (animal)	<input checked="" type="checkbox"/> Schwannoma	<input type="checkbox"/> Ras-independent
<input type="checkbox"/> Other	<input type="checkbox"/> Primary cells (human)	<input checked="" type="checkbox"/> Meningioma	<input type="checkbox"/> PI3K
	<input type="checkbox"/> Primary cells (animal)	<input checked="" type="checkbox"/> Optic Glioma	<input type="checkbox"/> Raf/MEK/ERK
	<input type="checkbox"/> Mouse models:	<input checked="" type="checkbox"/> Astrocytoma	<input type="checkbox"/> Rac 1/2/Rho
	<input type="checkbox"/> Transgenic	<input checked="" type="checkbox"/> MPNST	<input type="checkbox"/> PAK1
	<input type="checkbox"/> Human xenograft	<input checked="" type="checkbox"/> PNS Tumors - other	<input type="checkbox"/> mTOR
	<input type="checkbox"/> Other	<input checked="" type="checkbox"/> CNS Tumors - other	<input type="checkbox"/> PKCalpha
	<input type="checkbox"/> Animal models – other:	<input type="checkbox"/> Dysplasia/Bone Defects	<input checked="" type="checkbox"/> Other _____
	<input type="checkbox"/> Zebrafish	<input type="checkbox"/> Cardiovascular Defects	
	<input type="checkbox"/> Drosophila	<input type="checkbox"/> Cognition/learning	
	<input type="checkbox"/> Other _____	<input checked="" type="checkbox"/> Pain	
		<input type="checkbox"/> Blood disorders	
	Candidate therapeutics:	<input type="checkbox"/> Other _____	
	<input type="checkbox"/> Antibody		
	<input type="checkbox"/> Peptide		
	<input type="checkbox"/> Small molecule/chemical entity/array		
	<input type="checkbox"/> Gene therapy		
	<input type="checkbox"/> RNA silencing		
	<input type="checkbox"/> Other _____		

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Summary

The drug of interest is the neurotoxic heterodimeric phospholipase Crotoxin, isolated from the venom of the South American rattlesnake, *Crotalus durissus terrificus*. It has proven to have significant and broad acting anti-tumour activity both *in-vitro* and *in-vivo*. Its' increased affinity for cancer cells is due primarily to the target subunit (A, crotapotin) and cell death is brought about by the B subunit (Crotactine) through the membrane-disrupting enzymatic activity. Despite its potent neurotoxic activity it has significant potential as a therapeutic agent. Of major importance is the observation that Crotoxin induces tolerance to its neurotoxic effects without altering the cytolytic properties permitting the use of doses above that which would normally be lethal to the host. It has a higher lethal activity toward cancerous cells and spares normal cells presumably due to the altered make-up of the cell membranes produced by rapidly proliferating cells. The sensitivity of cancer cells to Crotoxin has been associated with the expression of epidermal growth factor, a surface receptor associated with malignancy. Cancer cell lines with the highest sensitivity to Crotoxin include lung, CNS, and melanoma.

There is a history of its' use in humans. The whole venom has been employed for decades as a pain reliever ultimately giving rise to the anticancer research. Several human safety trials were conducted with Crotoxin alone and in combination with cardiotoxin (VRCTC310) in patients with refractory cancer. The drug, administered by injection, was well tolerated in both forms, a maximum tolerable dose determined, pharmacokinetics and a side-effect profile established. Upon intramuscular injection Crotoxin achieves maximal circulating levels at 1 hour and is effectively cleared within 24 hours. Several severely ill patients appeared to respond to treatment with reduced tumour bioburden, amelioration of pain and improved "Quality of Life" being reported.

The reported pain relief was so consistent that the Sponsor engaged in laboratory studies to evaluate the product solely as a pain reliever. It produced antinociceptive activity equivalent to Demerol and was synergistic with aspirin. The most significant problem reported in these clinical studies was anaphylactoid reactions in two patients. The problem may have been controllable simply with the use of an antihistamine. Injection site reactions are frequent though they cease to occur approximately two weeks into the initiation of therapy. Interesting also are the observations that long-term therapy is feasible and free of immunological inhibition.

The identification of Crotoxin in 1938 has led to many publications on its activity and properties thereby being, in essence, a drug in development for decades. However, the focus of this clinical program is to evaluate the potential of Crotoxin and/or VRCTC-310 in the treatment of cancer. Although not fully understood, this mechanism involves the recognition of a set of structural elements present in the membrane of the target cells, which results in binding of the toxin. Structural perturbation of the membrane resulting from anchoring of the toxin and subsequent hydrolysis of membrane phospholipids leads to cell death. Thus Crotoxin differs from other cytotoxic agents in that:

- (1) *it appears to be a self-targeted toxin;*
- (2) *it exert its cytotoxic activity on the cell membrane of the target cells and*
- (3) *it is not an enzyme inhibitor, an antimetabolite and does not interacts with DNA.*

Crotoxin anti-tumour activity

Evaluation by the Developmental Therapeutics Program of the National Cancer Institute (NSC 624244) for cytotoxicity in vitro against a panel of human tumour cell lines showed enhanced cytotoxicity towards melanoma, CNS and non-small cell lung cancer lines. The COMPARE analysis of this pattern of specificity resulted unlike that of any know agent for which the mechanism of cell killing has been determined, suggesting a unique mechanism of action (Paull et al., 1989; Newman et al., 1993).

Crotoxin displays cytotoxic activity *in-vitro* against a number of murine and human tumour cell lines (Corin et al., 1993; Rudd et al., 1994). The cytotoxic effect of the unmodified toxin exhibited some degree of selectivity towards some tumour cell lines, since after 8 h incubation with 10 µg/ml Crotoxin, all the Hs87T (human breast ductal carcinoma) and Lu-1 (human lung adenocarcinoma) cells were killed while 20 % of 3T3-fibroblasts and 45% of normal human keratinocytes remained viable after 72 h. Morphologic alterations including blurred membranes and lysis of Hs 87T or Lu-1 cells are observed after 8 h incubation with Crotoxin at 2.3 µg/ml. After 72h the cell numbers were reduced to about 40% of the controls.

Studies on the cytotoxic activity of the Crotoxin complex and the isolated subunits on actively growing or quiescent murine erythroleukemia (MEL) cells indicated that subunit B is the only responsible for cytotoxicity., with an $EC_{50} = 1.5 \mu\text{g/ml}$. No cytotoxic activity was observed associated to isolated subunit A. Interestingly, the molar concentrations for EC_{50} with either Crotoxin complex or isolated subunit B were $9.8 \times 10^{-8} \text{ M}$ and $1.042 \times 10^{-7} \text{ M}$, respectively, suggesting that complex formation with subunit A does not inhibit the cytotoxic activity of subunit B. (Corin et al., 1993). MEL cells were incubated with 5.0 µg / ml Crotoxin. At different intervals of time samples were removed, an aliquot was used to count the cells, and the remaining cells were washed three times by centrifugation and inoculated into fresh medium supplemented with fetal bovine serum to test their ability to restart growth. After 1 h incubation no significant morphological alterations were observed, however only about 40% of the cells were able to restart growth. After 12 h incubation, there was cell lysis, accumulation of cell debris and most of the cells had severe morphological alterations, being unable to restart growth. This indicates that MEL cells die in less than a doubling time (18 h, see Corin et al., 1993)

The phospholipase A_2 activity of subunit B appears to be essential for cytotoxicity. Complex formation between subunit A and an inactive subunit B obtained by irreversible alkylation of the active site did not show cytotoxic activity on MEL cells (Corin et al., 1993), on A431, ME-180 (Donato et al., 1996) or OVCAR 3 cells (Alicea, 1998). Relationship between phospholipase A_2 activity and cytotoxicity was also demonstrated in OVCAR-3 cells by measuring the increase in concentration of free palmitic acid in the membranes of cells treated with Crotoxin (Alicea, 1998). Treatment of Hs 87T and OVCAR-3 cells with Crotoxin resulted in a time and concentration dependent increase in the number of cells staining with Trypan Blue (Rudd et al.,1994; Alicea, 1998). In addition, Crotoxin treatment produces the release of LDH activity to the medium, a marker of membrane damage (Alicea, 1998).

Examination of OVCAR-3 cells by scanning electron microscopy (Newman et al., 1996) showed direct evidence of membrane damage caused by exposure to Crotoxin. Treatment for 3 h already resulted in separation of the cells and the appearance of “white bodies” or blebs pushing the membrane from the cytoplasmic side. Longer periods of exposure revealed highly disrupted cell membranes showing “holes”. OVCAR-3 cells exposed to Crotoxin were also examined by transmission electron microscopy. The nucleus, endoplasmic reticulum and microvillie appeared well defined. However, there appeared a number of vesicles of similar sizes and shapes, increasing with the time of exposure, which were stained with Oil Red indicating the presence of lipids. In a recent review (Ownby et al., 1999) a model is presented to explain the difference between the effects of cytotoxic and non-cytotoxic phospholipases A_2 which is fully consistent with these observations (including vesicle formation).

The cytotoxic activity of Crotoxin can be prevented by adding anti-Crotoxin antibodies either to the toxin solution or to the medium. However, the addition of anti-Crotoxin antibodies 5-7 min after the addition of Crotoxin reduces cytotoxicity only poorly suggesting that either due to protein-lipid interaction, conformational changes and/or to covalent modification of the protein, the toxin becomes inaccessible for reaction with antibodies.

No difference in cytotoxicity were observed by the addition of 20 mM ammonium chloride or 0.5 mM quinacrine (inhibitors of the receptor-mediated endocytosis), suggesting that the toxin is not internalized. The protein-lipid interaction may be strong enough to induce structural perturbations in the membrane and to preclude a wash-out of the toxin. Damage may be further increased by continuous phospholipid hydrolysis, preventing membrane repair. Therefore, the cytotoxic effect of the toxin is, for all practical purposes, irreversible.

Sensitivity of malignant cells expressing EGFR

EGFR has emerged as a significant component in malignant disease. It represents one of the most important growth-regulatory signal-transduction molecules and exerts this function mainly through its' intrinsic tyrosine kinase activity, which can be activated upon ligand binding (Shin et al., 2001). It is frequently over-expressed in cancer cell lines. EGFR positivity may also be an indicator of poor prognosis.

Parental ME-180 cell line (ME-180 par, squamous endometrial carcinoma, human) was obtained from American Type Culture Collection (Rockville, MD). The derived variants ME-180 sen (sensitive to TNF) and ME-180 res (resistant to TNF) prepared by culturing the cells in the presence of increased concentrations of TNF, were established and characterized in terms of expression levels of EGFr (Donato et al., 1993). The A431 cell line (squamous vulvar carcinoma, human) was provided by Dr. G. Gallick (Department of Tumour Biology, MD Anderson Cancer Center). The ME-180 cell lines had similar generation times (22-28 h) but different levels of expression (both as protein and as mRNA) of epidermal growth factor receptor (EGFr). If the expression level of EGFr in ME-180 par cells or ME-180 sen cells is taken as 1.0, that of ME-180 res will be 5.0 and that of A431 cells will be 10.0.

Incubation with Crotoxin showed a 60-fold difference in sensitivity to cytotoxic action. The cells expressing high densities of EGFr (A431 and ME-180 res) were the most sensitive to the cytotoxic action (EC_{50} 1.5 $\mu\text{g/ml} = 6.3 \times 10^{-8}$ M) while the less sensitive were those with lower density of EGFr (ME-180 par and ME-180 sen, EC_{50} 110.0 $\mu\text{g/ml} = 4.6 \times 10^{-6}$ M). This specificity is not observed using other cytotoxic agents like *Naja naja atra* cardiotoxin isoform III, which killed all these cell lines with EC_{50} 20 $\mu\text{g/ml}$. Correlation between EGFr expression density and sensitivity to Crotoxin induced cytotoxicity is supported by other evidence: (a) pretreatment for 4 h of A431 cells with EGF (2.0 nM) which decreases the levels of EGFr expression (down regulation) also reduces the antiproliferative effect of Crotoxin; (b) the sensitivity to Crotoxin induced cytotoxicity (measured as EC_{50}) of a variant of ME-180 res line, which do not overexpress EGFr is similar to those of ME-180 par and ME-180 sen.

In addition, the correlation between EGFr expression density and sensitivity to Crotoxin mediated cytotoxicity was observed in other tumour cell lines. The cell line DiFi (colon adenocarcinoma, human) overexpress EGFr (by about 100-fold) respect to the cell line HT-29. The EC_{50} for Crotoxin mediated cytotoxicity is 2.0 $\mu\text{g/ml}$ for DiFi cells and 44.0 $\mu\text{g/ml}$ for HT-29 cells (Donato et al., 1996).

Evaluation by the DTP (NCI) for in vitro cytotoxicity of Crotoxin against human tumour cell lines indicated that ovarian cancer cell lines were quite resistant. In fact, the EC_{50} of Crotoxin against the OVCAR-3 cell line is 116 $\mu\text{g/ml}$ (6.9×10^{-6} M, Alicea, 1998). The variant cell line OVCAR-3 R-3 resistant to TNF and cisplatin was obtained by culturing the parental OVCAR-3 line in the presence of increasing concentrations of TNF, it exhibits a high EGFr expression level, and the EC_{50} for Crotoxin is 16.4 $\mu\text{g/ml}$ (6.8×10^{-7} M, Alicea, 1998). Comparative measurement of cell damage such as incorporation of Trypan blue, release of LDH or increase in free palmitic acid in the membrane indicated that for similar Crotoxin concentrations, the damage induced in the OVAR-3 R-3 was much higher than in the parental OVCAR-3 cell line (Alicea, 1998)

Both the phospholipase A_2 activity and dissociation of the Crotoxin complex appear to be required for the antiproliferative activity of Crotoxin on the sensitive cell lines. The selective alkylation of the His-48 residue in subunit B or covalent cross-linking of the Crotoxin subunits (which maintains phospholipase A_2 activity but prevents dissociation of the Crotoxin complex into their subunits) completely abolish Crotoxin mediated cytotoxicity.

Interestingly, preincubation of A431 cells with Crotoxin at concentrations close to the EC_{50} did not significantly affect the binding of radiolabeled effector (^{125}I -EGF) to the EGFr, indicating that the relationship between Crotoxin-mediated cytotoxicity and EGFr overexpression do not involve the effector binding domain. (Donato et al., 1996). Further studies on A431 cells or cell membranes showed that incubation with Crotoxin did not interfere with the binding of EGF or the recovery of EGFr.

Incubation of cell membranes from A431 cells treated with Crotoxin with ^{32}P -ATP followed by separation of phosphorylated proteins by SDS-PAGE showed a 3-fold increase in EGFr phosphorylation, which

was time and concentration dependent. A similar result was obtained by immunoprecipitation of the EGFr from cell membranes treated with Crotoxin. Pretreatment with EGF also results in increased tyrosine kinase activity of the EGFr, and Crotoxin mediated increase in tyrosine phosphorylation of the EGFr is additive to that produced by EGF. Again, this effect requires the phospholipase A₂ activity of Crotoxin. (Donato et al., 1996)

Crotoxin mediated modulation of the tyrosine kinase activity of EGFr can also be shown on intact A431 cells. However, Crotoxin treatment did not increase phosphorylation of the EGFr in ME-180 par or ME-180 sen cells, which expressed low levels of EGFr (Donato et al., 1996). The relevance of Crotoxin mediated modulation of the tyrosine kinase activity of EGFr in Crotoxin induced cytotoxicity is not clear. In fact, toxin-phospholipid interaction and subsequent accumulation of products of phospholipid hydrolysis in the membrane may alter membrane packing, disrupt lipid domains and affect protein conformation resulting in effects like inhibition of type II Ca²⁺ channels or alteration of transmembrane signaling pathways. However both, Crotoxin mediated increase in EGFr phosphorylation and cytotoxic activity require phospholipase A₂ activity and dissociation of Crotoxin complex, and both are observed in cell lines sensitive to Crotoxin cytotoxicity *only*. Interestingly, the increase in tyrosine kinase activity of EGFr is usually considered to be a *growth signal*. On the other hand, dysregulation or untimely activation of these signals in EGFr hyperproducing cells may result in reverse signaling and growth suppression or cell death. (Hirai et al., 1988; Nishikawa et al., 1994). No evidences of Crotoxin induced apoptosis (DNA fragmentation, PS transposition) were observed in either parental or EGFr hyperproducing cell variants (Alicea, 1998).

Crotoxin in-vivo studies

Studies on animal models were performed using murine and human tumour cell lines. Tumour cell suspensions (>90 % viable) were inoculated (1 x 10⁵ to 1 x 10⁶ cells per animal) either subcutaneously, intraperitoneally or intracerebrally. Tumour fragments were implanted subcutaneously. At day 7 after inoculation (day 3 after implant), Crotoxin was administered by means of daily injections at doses of approximately 0.04 mg/kg (mice, i.p.); 0.08 mg/kg (mice, i.m.); 0.2 mg/kg (rats, i.p.) or 0.4 mg/kg (rats i.m.). Treatment continued for 30 to 60 days, depending on whether the experiment end-point was increase in life span (ILS %) or growth inhibition (GI, %). Treated animals which survived or had 100% GI were kept for 2-3 months without treatment, and then sacrificed for pathologic examination. Only when the pathology report was negative, they were qualified as complete responses (CR). A summary of some studies is tabulated below (table 1).

Table 1. Summary of Crotoxin effects in-vivo.

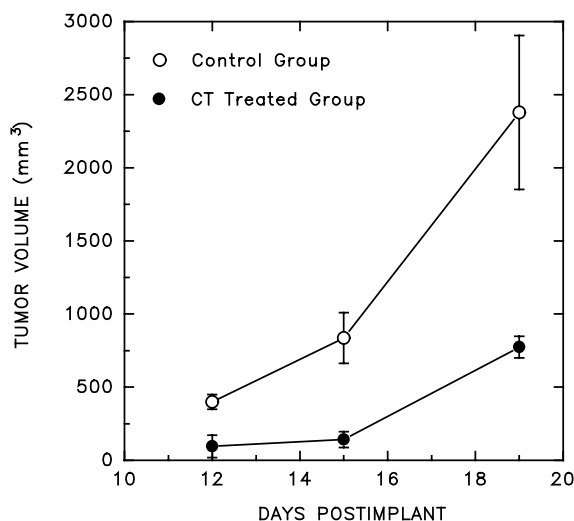
Tumour	Implant or inoculum	Host	GI (%)	ILS (%)	CR (%)
MET-A ^a	1 x 10 ⁶ cells, i.p.	BDF ₁ mice	-	33	-
	1 x 10 ⁵ cells, i.p.	BDF ₁ mice	-	indefinite	>90
B-16 melanoma	2 x 10 ⁶ cells, i.p.	C-57 Bl mice	-	25	-
	2 x 10 ⁵ cells, i.p.	C-57 Bl mice	-	62	-
Lewis lung carcinoma ^b	fragments	BDF ₁ mice	83	-	12
Walker 256 ^c carcinosarcome	1 x 10 ⁶ cells, i.p.	Sprague-Dawley rats		67	20
MX-1 (human ductal ^d carcinome)	fragments	SCID mice	68	-	-
	fragments	Nude rats	89		
EO-1 (human ^e oligodendroglioma)	2 x 10 ⁶ cells, s.c.	SCID mice	65		
	1 x 10 ⁶ cells, s.c.	SCID mice	75		
	1 x 10 ⁵ cells, i.c.	SCID mice		40	-

Notes:

- ^a . With MET-A at 1×10^5 cells/mouse, 11 out of 12 animals survived 2-3 months without signs of disease.
- ^b Performed in parallel with Dr. R.A. Newman, (The University of Texas, MD Anderson Cancer Center., Houston, TX).
- ^c No “no takes” were detected, as shown by the observation of carcinosarcoma cells in peritoneal fluid on days 7 and 10. Surviving animals (20%) did not show signs of disease 2 months after inoculation.
- ^d Performed in parallel with Dr. D. Dykes, (Southern Research Institute, Birmingham, AL).
- ^e SCID mice were provided by the Edwin L. Steele Laboratory for Radiobiology, of the Massachusetts General Hospital, Boston, MA).

The *in-vivo* antitumour activity of CT (Fig. 1) has shown impressive results against a lung carcinoma, as illustrated below. Here, animals were treated on study days 1-2, 5-7, and 12-14. The maximum growth inhibition was obtained on day 15 (one day after the end of treatment) and was 83%.

Figure 1. In vivo antitumour activity of CT against the murine Lewis lung carcinoma



To examine the antitumour action of CT vis-a-vis other chemotherapeutic agents it was compared to preclinical *in vivo* results on Lewis lung carcinoma obtained by the NCI for standard anticancer agents (Table 2). Results obtained with Lewis lung carcinoma were compared by the DTP, NCI with those obtained with other agents. Crotoxin ranked third, after cyclophosphamide and cisplatin in the list of the more potent antitumour agents against Lewis lung carcinoma.

Table 2. Comparison of Crotoxin to other chemotherapeutic agents.

Agent	Regime	Dose (mg/kg)	GI (%)
Cyclophosphamide	d1	180.0	100
Cisplatin	d1-11	2.4	96
Crotoxin	d1-19	0.067	84
Methrotexate	q4d x 3	65.0	80
	d1-9	1.8	60
Adriamycin	q4d x 3	3.0	65
Vincristine	d1-9	0.14	54
Melphalan	d1-11	3.0	53
Vinblastin	d1-11	0.25	28

Induction of Neurotoxic Tolerance

Mice injected daily with progressively increasing doses of crotoxin develop tolerance to the lethal action of this toxin. Treated mice tolerated daily doses of crotoxin 20- to 35-fold higher than the original LD50 without the characteristic signs of toxicity. Studies on the isolated phrenic nerve-diaphragm preparation in vitro from control (crotoxin-naive) mice showed that the exposure to 2 to 10 micrograms/ml crotoxin in the bath produced complete transmission blockade in 120 to 150 min. Conversely, the preparations from crotoxin-treated mice required crotoxin concentrations in the range of 17.5 to 100 micrograms/ml to produce complete neuromuscular block, being virtually insensitive during 200 min of exposure to 5 to 10 micrograms/ml crotoxin. Phrenic nerve-diaphragm preparations of control (crotoxin-naive) and crotoxin-treated mice did not show significant differences in sensitivity to the blocking action of carbamylcholine, suggesting that induction of tolerance to crotoxin is likely a presynaptic event (Okamoto et al., 1993).

In the course of anti-tumour studies, tolerance was developed to the neurotoxic properties of VRCTC-310 that allowed comparison of several schedules of fixed and escalating daily i.m. doses to mice bearing s.c. Lewis Lung carcinoma. An 83% inhibition of tumor growth was achieved using an escalating dose schedule starting at 1.8 mg/kg and reaching 6.3 mg/kg/day on day 20. Although some irritation around the sites of i.m. injection was noted, animal weight loss was negligible and there were no other signs of adverse toxicity (Newman et al., 1993). The interest of this phenomenon resides in the possibility on whether tumour cells may become "tolerant" to VRCTC-310. The antitumour activity of VRCTC-310 on Lewis lung carcinoma implanted in "tolerant" mice showed tolerance to VRCTC-310 did not affect tumour growth in control "tolerant" mice, however growth inhibition appeared to be higher since larger doses of VRCTC-310 could be administered (Newman et al., 1993).

Analgesic effects of Crotoxin

This study investigated analgesia induced by crotoxin and the effects of atropine and Naloxone on antinociceptive actions of crotoxin in mice and rats. The results showed that crotoxin at 66.5, 44.3, 29.5 µg/kg (ip) exhibited a dose-dependent analgesic action in mice using hot plate test and acetic acid writhing test. The peak effect of crotoxin's analgesia was seen 3 h after its' administration. Crotoxin at 44.3 µg/kg (ip) had significant analgesic action in rat tail-flick test. In the mouse acetic acid-writhing test, intra-cerebral ventricle administration of crotoxin 0.3 µg/kg (1/130 of systemic dose) produced marked analgesic effects. Microinjection of crotoxin (0.15 µg/kg) into the periaqueductal gray area also elicited a robust analgesic action in rats with hotplate test. Atropine at 0.5 mg/kg (im) or 10 mg/kg (ip) or Naloxone at 3 mg/kg (ip) failed to block the analgesic effects of crotoxin. These results suggest that crotoxin has analgesic effects and the site of antinociceptive activity appears to be situated within the central nervous system. The cholinergic system and the opioid system appear not to be involved in antinociceptive actions of crotoxin (Zhang et al., Toxicon 2006, in publication).

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Human Studies

Both crude and purified forms of Crotoxin have been through at least 4 clinical investigations. The results of five of these studies have been published in scientific journals. The results from each condensed into table 1 and briefly summarized below. While Crotoxin is the principle active agent of interest the drug has been employed alone and in combination with Cardiotoxin (VRCTC-310), an agent that enhances the activity of Crotoxin *in-vitro* (by about 2-fold). There is no strong evidence that Cardiotoxin has such activity *in-vivo* despite its' published and unpublished potent anti-tumour effect *in-vitro*. Consequently, studies conducted under the FDA-based IND elected to employ Crotoxin solely as the API. At the doses used, crotoxin administration did not affect hematopoietic, hepatic, or renal functions. This coincides with results from preclinical studies in dogs (DeTolla et al., 1995).

Table 1. Human Studies.

No. of patients	Duration	Dosage	Route	ADR
15	30 days	0.0025mg up to 0.023 mg/Kg daily	i.m.	2 anaphylactic reactions
2	6 weeks	0.14mg/Kg/week	i.t. & p.t.	None reported
5	8 weeks	0.014 mg/Kg/week	i.t. & p.t.	Not available
23	30 days longest 117 days	0.03-0.22mg/m ² daily	i.m.	1 anaphylactic reaction
1	2.5 years	0.09mg to 1mg per dose	i.t., s.c. & i.v.	Transient injection site reactions

i.t. = intratumour, p.t. = peri-tumourly

Naturally the most important aspect in drug development pertains to safety and, to a lesser degree, the risk/benefit ratio if that can be reasonably assessed. Crotoxin has demonstrated relatively low toxicity and a therapeutic window that instills confidence in its use. Over this half century many investigators, both academic and clinical, animal and human, have observed the effects of this drug in numerous laboratory studies. There is a history of its' use in humans. The whole venom has been employed for decades as a pain reliever ultimately giving rise to the anticancer research. Several human safety trials were conducted with Crotoxin alone and in combination with Cardiotoxin (VRCTC310, comparator product) in patients with refractory cancer. The drug, administered by injection, was well tolerated in both forms, a maximum tolerable dose determined, pharmacokinetics and a side-effect profile established. Upon intramuscular injection Crotoxin achieves maximal circulating levels at 1 hour and is effectively cleared within 24 hours. Several severely ill patients appeared to respond to treatment with reduced tumour bioburden, amelioration of pain and improved "Quality of Life" being reported.

Table 2. Cancer types observed in participating Patients.

Publication reference	Year	Product	Cancer types involved
Plata et al.	1992	Crotoxin (Patent)	Adenocarcinoma of the lacrimal sac with metastasis Infiltrating ductal carcinoma with metastasis Mixed glioma Pancreatic adenocarcinoma Pancreatic tumour Peritoneal mesothelioma Undifferentiated fusocellular sarcoma
Costa et al.	1997	VRCTC310	Gastrointestinal Breast Lung Laryngeal Maxillar Cervical Ovarian
*Costa et al.	1998	VRCTC310	Squamous cell carcinoma Chordoma
Costa et al.	1998	VRCTC310	Breast cancer Squamous skin carcinoma
Cura et al.	2002	Crotoxin	Gastrointestinal cancer NSCL Squamous cervix carcinoma Thyroid carcinoma Larynx carcinoma Bladder carcinoma Fallopian tube adenocarcinoma Head and neck carcinoma Low grade fibrocarcoma Ewing's sarcoma Liposarcoma
Reid (unpublished)	2003	Crotoxin	Malignant histiocytoma with metastasis

The reported pain relief was so consistent that the Sponsor engaged in laboratory studies to evaluate the product solely as a pain reliever. It produced antinociceptive activity equivalent to Demerol and was synergistic with aspirin. The most significant problem reported in these clinical studies was anaphylactoid reactions in two patients. This aspect is also interesting in the light of studies suggesting Crotopotin has immunosuppressive properties. Injection site reactions are frequent though they cease to occur approximately two weeks into the initiation of therapy. Interesting also are the observations that long-term therapy is feasible and free of immunological inhibition. Generally, it seems that many snake products do not induce strong neutralizing immune responses even if resultant antibody responses are high though this aspect warrants further study with respect to Crotoxin.

In order to minimize allergic reactions to patients the general approach is to employ a ramping rate of drug administration preferably extending over a period of 2 weeks, a critical time in the development of antibodies. While antibodies can block the cytotoxic activity of the drug(s) it seems that in patients long-term treatment can be accomplished suggesting either immunosuppression, the development of non-neutralising antibodies or immune tolerance. It is believed that the reported anaphylactic reactions may be suppressed by the use of Benadryl. The injection of detoxified venoms did not cause anaphylactic reactions though significant injection site reactions could be observed. Benadryl was employed to control these reactions.

Intramuscular injections certainly provoke the production of an immune reaction and may not necessarily be the optimal method for administration hence the decisions to examine i.v. administration. Upon

injection there is some minor pain. Approximately 20-30 min post-injection swelling, erythema and pruritus appear similar to that of an insect sting. This reaction may endure for over 24 hours. The myonecrotic activity of the drug will induce tenderness at the injection site that will continue for several days. If the patient can overcome this reaction the injection site reactions will slowly subside over the course of 2-3 weeks at which point no injection site reaction will occur. However, if the patient ceases the use of Crotoxin for periods over 3 months it will require a new round of inducing injection tolerance. While there are some concerns over damage to kidney cells the i.v. route of administration (using an indwelling catheter) provides the most convenient and comfortable method of administering the drug. This aspect will be closely monitored in the proposed study. This approach permits the patient to be treated on an out-patient basis following training on the drug administration.

Conveniently, the first symptom of over-dosing (toxicity) is diplopia, associated with bolus doses in non-tolerant patients of 0.5mg. The effect is completely reversible, subsides 2-3 hours after onset and resides far below a true toxic dose of Crotoxin (LD₅₀ estimated at 60 ug/Kg). With the induction of tolerance the potential for reaching a toxic dose is greatly reduced. As Crotoxin clears rapidly from the host a multi-dosing schedule can be employed to administer more drug without obtaining indications of toxicity.

Generally, it has been found that Crotoxin does not induce long-term neuropathy observed with radiation therapy nor does it induce the nausea, hair loss and weight loss associated with chemotherapy. The drug does not induce the malaise associated with the use of interferons and its broad specificity is superior to monoclonal antibodies. The drug can be employed on a transient basis and reused when required. It is cytotoxic unlike the developing angiogenesis products but spares normal cells. The proteinaceous nature of the drug makes the metabolic products of the drug regarded as harmless peptides. Responses to the drug have been observed within a month of treatment and chronic treatment for long periods is possible.

2.3.5 References

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