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VRCTC-310 – A novel compound of purified animal toxins separates antitumor efficacy from neurotoxicity

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Summary

Two purified animal venom toxins, crotoxin and cardiotoxin, have been combined to produce a unique natural product (VRCTC-310) currently under investigation as an antitumor agent by the National Cancer Institute. *In vitro*, it has demonstrated cytotoxic disease specificity and a unique mechanism of action when submitted to COMPARE analysis. *In vivo*, tolerance was developed to the neurotoxic properties of crotoxin which 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. This natural product represents a new, membrane interactive anticancer agent which produces a unique spectrum of cytotoxicity *in vitro* and which has demonstrated interesting *in vivo* antitumor efficacy.

Introduction

Venoms from numerous land snake species have been shown to possess cytotoxic and/or lytic effects on tumor cells in culture [1,2]. Of interest are several reports that certain membrane-active components from cobra venoms have been found to be cytotoxic to tumor cells at concentrations that are significantly lower than those required to produce similar effects in normal cells [3,4]. Recent evidence indicates that some PLA_2 enzymes from snake venom can bind to cell membranes and produce cytotoxicity [5]. The possibility of achieving selective cytotoxicity in malignant cells with an isolated PLA_2 by means of specific membrane-receptor binding and subsequent enzymatic hydrolysis of membrane phospholipids would present a unique approach to anticancer therapy.

Because of the complex composition of many venoms and the inherent toxicity associated with them, their antitumor efficacy in mice has been difficult to evaluate. Recently, we investigated the cytotoxicity of crotoxin against murine erythroleukema cells [6]. We have also reported that mice can be made tolerant to the neurotoxicity of crotoxin [7]. This observation made it possible to investigate other pharmacodynamic properaties (*e.g. in vivo* antitumor efficacy) of the purified venom toxin at higher doses.

VRCTC-310, a novel mixture of a noncovalent heterodimer, crotoxin (CT) complex, and a basic amphipathic peptide, cardiotoxin (CD), is currently under preclinical evaluation as an antitumor agent at the National Cancer Institute. CT, a PLA_2 neurotoxin purified from the venom of the South American rattlesnake *Crotalus durisuss terrificus* [8], can be dissociated into two non-identical subunits. Subunit A is an acidic polypeptide of 82

amino acid residues (9.5 kDa). Subunit B, a basic phospholipase A_2, is formed by a single polypeptide chain of 122 amino acid residues (14.5 kDa). CD is a highly basic (isoelectric point >11) membrane disruptive peptide from the venom of the Taiwanese cobra *Naja naja atra*. CD consists of a single polypeptide chain of 60 amino acid residues (7.0 kDa) [9]. These toxins were evaluated for *in vitro* antitumor activity by the Developmental Therapeutics Program, Division of Cancer Treatment, of the National Cancer Institute. Separately tested, CT and CD were cytotoxic bud did not meet the established institutional criteria of disease specificity. However, a combination of CT and CD was found where cytotoxicity and selectivity towards certain tumor cell lines was significantly enhanced.

While the biochemistry and toxicology of the separate CT and CD have been extensively studied [9, 10], their combination results in a product (VRCTC-310) with unusual pharmacologic properties. CD increases the PLA_2 activity of CT by twofold when it is present in a molar ratio of 3:1 (CD:CT). This specific ratio also results in up to a 15-fold enhancement of the relative *in vitro* cytotoxicity of CT.

The increased cytotoxicity of CT, however, is accompanied by an actual decrease in CT-mediated neurotoxicity. Although the mechanism(s) that aid mice in developing tolerance to the neurotoxic properties of CT are not fully understood, the ability to eliminate lethal neurotoxicity enabled an evaluation of antitumor efficacy in an *in vivo* tumor model. In this report we present data indicating that VRCTC-310 has a broad spectrum of cytotoxicity against tumor cells *in vitro* and has antitumor activity against a solid tumor model in mice. Further, this activity increases under higher dose regimens when mice are made tolerant to the neurotoxic properties of this unique combination of venom products.

Materials and methods

Isolation and purification of venom components

CT was purified from the venom of *Crotalus durissus terrificus* (Instituto Butantan, Sao Paulo, Brazil) by chromatography on Sephadex G-75 [12] and chromatography on DEAE-Sephadex A-50 as described by Aird and Kaiser [13]. CD, Fraction III, was purified from the venom of *Naja naja atra* (Miami Serpentarium, Punta Gorda, Florida) according to Yang *et al.* [14]. The purity of the two components was assessed by PAGE (polyacrylamide gel electrophoresis) in nondenaturing media and by SDS (sodium dodecyl sulfate)-PAGE and by analysis of amino acid composition. Solutions of CT and CD were prepared separately in phosphate-buffered saline (PBS) and sterilized by filtration. The protein concentrations were determined by spectrophotometric analysis using the molar absorptivities of $E_{278} = 41000\ M^{-1} \cdot cm^{-1}$ for CT [15] and $E_{276} = 4500\ M^{-1} \cdot cm^{-1}$ for CD [16]. The appropriate volumes of CT and CD solutions were mixed to obtain a molar ratio of 3:1 (CD:CT) and the mixture, designated VRCTC-310, was diluted with PBS to the desired final concentrations.

Determination of cytotoxicity of CT, CD and VRCTC-310 in an in vitro human tumor cell line assay

CT, CD, and various mass ratios of these two materials (including VRCTC-310) were evaluated by the National Cancer Institute for cytotoxicity against an *in vitro* panel of human tumors as previously described [11]. The tumor panel consisted of 60 tumor cell lines organized into seven subpanels which included leukemia, lung (Non-Small Cell and Small Cell), colon, renal, ovary, melanoma, and brain tumors. Each cell line was inoculated, preincubated on microtiter plates and solutions of CT, CD or VRCTC-310 in concentrations ranging from 158 μg/ml to 0.0158 μg/ml, in 1-log dilutions, were added and the culture incubated for 48 hours. Endpoint determinations of growth inhibition were made using sulforhodamine B.

Animals

Male mice ($B6D2F_1$ and $C_{57}BL/6$, 4 weeks old at the beginning of experiments) were obtained from

Table 1. Study I escalating dose schedules for VRCTC-310 dose (mg/kg)

Day	Non-tolerant mice	Day	CT-tolerant mice
1 to 2	0.135	1 to 2	1.80
3	0.180	3	2.25
4	0.225	4	2.70
5 to 8	0.270	5	3.15
9	0.315	6	3.60
10 to 11	0.360	7	4.05
12	0.450	8	4.50
13–16	0.540	9	4.95
17	0.630	10 to 18	5.40
18	0.720	19	6.30
19	0.810		

Taconic Laboratories (Germantown, NY) and kept in a temperature controlled environment with 12-hour light/dark cycles. They received food and water *ad libitum*. The LD_{50}s of CT, CD and VRCTC-310 were determined by different routes of administration following the procedure of Meier and Theakston [17].

Induction of tolerance

Mice treated daily with CT at increasing doses become tolerant to its neurotoxic action [7], thus allowing administration of doses of VRCTC-310 that would otherwise be lethal. Similarly, tolerance to the neurotoxic action of CT can be achieved by treating mice with progressively increasing doses of VRCTC-310 (Viskatis, LJ, Vidal, JC, Etcheverry, MA, unpublished results). In order to determine antitumor activity at higher doses of VRCTC-310, desensitization to the neurotoxicity of this product was performed in two schedules, namely (a) by progressively increasing the daily dose of VRCTC-310 during the course of antitumor treatment (*i.e.* after tumor implant), or (b) by desensitizing mice to CT before implanting the tumors, then initiating treatment with VRCTC-310 on an escalating- or fixed-dose schedule.

In study I, tolerance was induced using both methods, *i.e.*, in the escalating low-dose group, mice were not treated with CT before being given escalating doses of VRCTC-310. In the escalating high-dose group, however, mice were made tolerant to VRCTC-310 through a series of escalating daily intraperitoneal (i.p.) injections of CT (from 0.014 to 0.27 mg/kg) for 18 days, before initiation of antitumor therapy. In study II, mice were made tolerant to a higher dose of VRCTC-310 by receiving a longer CT treatment before tumor implantation and antitumor therapy (*i.e.*34 days of daily i.p. injections of CT from 0.014 to 1.26 mg/kg). Control animals were also pretreated to show that induction of tolerance before tumors were implanted had no effect on tumor growth.

Antitumor efficacy testing of VRCTC-310

Samples of Lewis lung carcinoma were a kind gift of Dr. Donald J. Dykes of the Southern Research Institute (Birmingham, Alabama). Tumor was propagated in C_{57}BL/6 mice until it was implanted into the study B6D2F1 mice. Tumor implants consisted of 8 mm^3 fragments placed subcutaneously (s.c.) in the upper back region of mice. In study I, an intramuscular (i.m.) fixed dose (0.23 mg/kg) of VRCTC-310 was compared with two different escalating-dose regimens (see Table 1). The escalating i.m. low-dose regimen was followed for nontolerant mice and started at 0.135 mg/kg VRCTC-310. Doses were progressively increased to 0.81 mg/kg over 20 days. The escalating i.m. high-dose regimen was possible because CT tolerance had already been induced. The treatment schedule for this group began at 1.8 mg/kg VRCTC-310 and was progressively increased to 6.3 mg/kg over the 20-day therapy period. In study II, mice received a longer pretreatment to allow a starting treatment dose of 4.5 mg/kg (Table 2). Tumor size was assessed at least every 3 days starting between days 8 and 10 after implant when the largest tumor diameters were about 10 mm. Tumor volumes were calculated by measuring the major (A) and minor (B) perpendicular diameters of the tumor in millimeters and by applying the formula: volume = $0.5\ (A \times B^2)$.

Table 2a. Study II pre-treatment with CT

Day	Dose (mg/kg)	Day	Dose (mg/kg)	Day	Dose (mg/kg)
1–2	0.014	13	0.077	23	0.540
3	–	14	0.090	24	0.720
4	0.018	15	0.110	25–26	0.900
5	0.023	16	0.113	27–28	0.900
6	–	17	0.135	29	1.080
7–8	0.027	18–20	0.180	30	–
9–10	0.045	21	0.270	31	1.260
11–12	0.063	22	0.360	32	–
				33	1.260
				34	–

Table 2b. Treatment dose schedule for study II

Day	Dose (mg/kg)
1 to 12	4.5
13 to 15	5.0
16 to 19	5.5
20 to 22	6.0
23 to 25	6.5

Data analysis

Data are presented as mean ± standard error of the mean unless otherwise stated. The significance of the differences in tumor growth was assessed by applying the f-test to pairs of groups [18] to the tumor sizes on the days of measurment. When variances were homogeneous, Student's T-test was applied and when the variances were heterogeneous, Cochran's T-test was applied [19]. *In vitro* data were evaluated using both the COMPARE statistical computer program [35] and an isobologram analysis to determine the optimal ratio of CD to CT.

The COMPARE program facilitates the detection, ranking, display and analysis of patterns of growth inhibition of cytotoxic agents. It has the potential of relating patterns of growth inhibition to specific drug-mediated mechanisms involved in inhibition of cell growth. The *in vitro* data were also subjected to isobologram analysis. Varying ratios of CT and CD (including the mass weight ratio of 1:1, VRCTC-310) were examined for their relative abilities to produce inhibition of cell growth in all cell lines tested.

Table 3. Comparison of cytotoxicity of CT, CD and VRCT-310 (LC_{50} μg/ml)

	CD	CT	VRCTC-310
LEU	6.3	43.6	7.2
NSCLC	3.9	12.7	5.0
SCL	1.2	30.1	3.9
COL	22.4	74.9	36.4
CNS	2.2	4.0	2.1
MEL	1.9	7.6	2.8
OVA	5.4	21.0	7.3
REN	3.2	10.1	5.4

The average LC_{50} (μg/ml) for each subpanel (lung subpanel divided into Non-small cell and Small cell) is given for CD alone, VRCTC-310, CT present in the VRCTC-310 product, and CT alone. Tumor abreviations: LEU = leukemia, NSCLC = non-small cell lung cancer, SCL = small cell lung, COL = colon, CNS = central nervous system, MEL = melanoma, OVA = ovarian, and REN = renal.

Results

In vitro experiments

By determining the average LC_{50} for all cell lines tested and considering the difference from the average for each tumor subpanel, a pattern of tumor sensitivity was obtained (Table 3). CT was cytotoxic to all cell lines at doses ranging from an average of 74.9 μg/ml for the colon to 4.0 μg/ml for the CNS (central nervous system) tumors. The average LC_{50} for all cell lines was 25.5 μg/ml. CD was also cytotoxic to all cell lines, the colon again being the least sensitive (22.4 μg/ml) and the melanoma the most sensitive (1.9 μg/ml). The average LC_{50} for

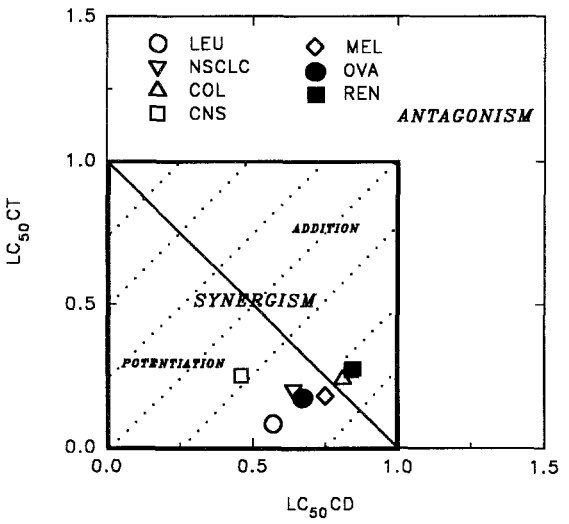

Fig. 1. Effect of dose combination of CT and CD *in vitro*. Data from experiments presented in Table 3 are plotted to show synergistic effect of the combination of CT and CD. Axes represent the relative LC_{50} doses of the separate components CT and CD to which the combined product, VRCTC-310, was compared. The effect of the combination dose of CT and CD represented by symbols inside the rectangle with diagonal lines shows that the compounds are synergistic.

all tested cell lines was 5.8 μg/ml. VRCTC-310 was cytotoxic to cells at concentrations as great as 2- to 6-fold lower than CT alone. It exhibited enhanced tumor cell killing for the CNS, melanoma and to some extent the lung (Small Cell Lung) subpanels. The average LC_{50} value for this assay was 3.34 μg/ml (1.67 μg/ml CT + 1.67 μg/ml CD). VRCTC-310 resulted in a synergistic potentiation of CT cytotoxicity of up to 15-fold (Small Cell Lung). Figure 1 illustrates the combined synergistic effect of CT and CD.

The diagonal line in the figure indicates the cytotoxic action of the CT and CD products which would be expected if their combined actions were merely additive. The fact that for at least 5 of the tumor cell line types examined in this study the observed average LD_{50} values fall below this line is indicative of synergism. In separate studies different ratios of CT to CD were examined for their ability to produce maximal cytotoxic effects. A CT:CD mass ratio of 1:1 (VRCTC-310) was found to result in maximal cell growth inhibition in comparison to CT:CD ratios of up to 4:1 and CD:CT ratios up to 4:1 (data not shown).

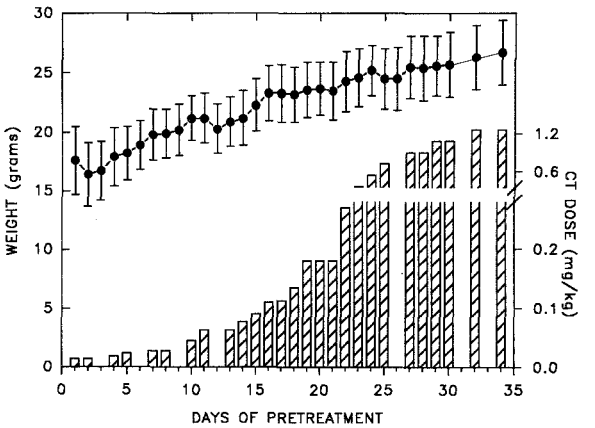

Fig. 2. Induction of tolerance to CT in B6D2F1 mice. Data are presented as mean body weight ± standard deviation (-0-) versus day of treatment. Doses of CT are indicated by hatched bars; N = 10.

Induction of tolerance

Mice injected with CT become tolerant to its neurotoxicity [7.] Although a minor loss of body weight was noted after the initial i.p. injection of CT (0.014 mg/kg), subsequent injections of doses up to 1.26 mg/kg were achieved without significant toxicity (Fig. 2). When individual mice showed evidence of more than a 10% loss of starting body weight, a delay of 1 or 2 days was sufficient to permit continued escalation of doses without further evidence of toxicity. As Table 1 shows, it was possible to achieve tolerance to as much as 6.3 mg/kg VRCTC-310, a dose that is approximately seven times the single i.m. LD_{50}. The LD_{50} of VRCTC-310 in mice after a single i.p. injection is approximately 0.45 mg/kg; the LD_{50} of one i.m. dose is 0.93 mg/kg. When compared with the i.p. LD_{50} of CT (0.095 mg/kg) and CD (1.48 mg/kg), the combined CT and CD product (VRCTC-310) results in a 2.5-fold decrease in CT-mediated neurotoxicity. Thus, the addition of the non-neurotoxic CD in a molar ratio of 3:1 (1:1 in mass) with respect to CT provides protection against the neurotoxic effects of CT.

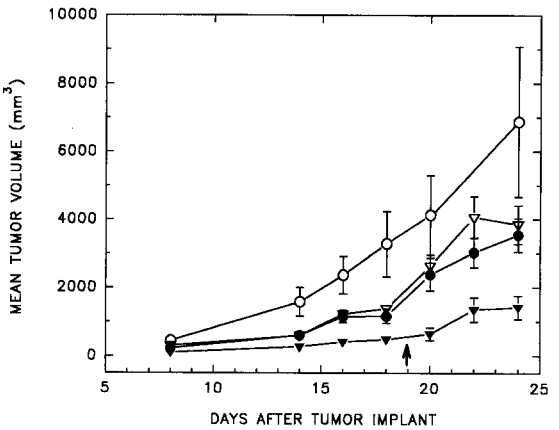

Fig. 3. Study I. Antitumor efficacy of VRCTC-310 in nontolerant and tolerant B6D2F1 mice bearing s.c. Lewis lung carcinoma. Data are presented as mean tumor volumes in the control (-0-), fixed (- ▽ -), escalating (-0-), and tolerant escalating doses (- ▼ -) for groups of 10 mice each. Doses and injection schedules are in Materials and Methods section. The arrow indicates the final day of VRCTC-310 injection.

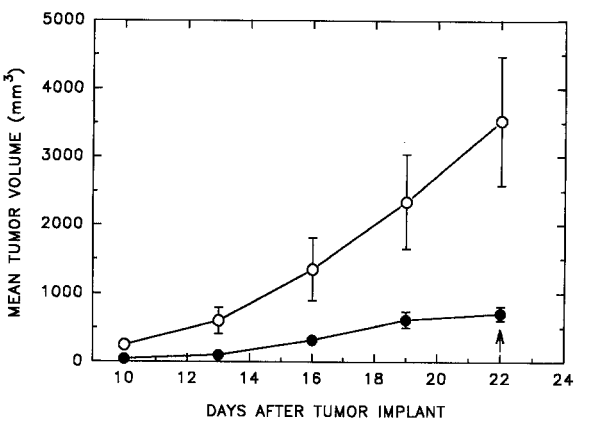

Fig. 4. Study II. Antitumor efficacy of VRCTC-310 in tolerant B6D2F1 mice bearing Lewis lung carcinoma. Data are presented as mean tumor volumes ± standard error of the mean in the control (-0-) and tolerant escalating high dose (-0-) groups; there were seven mice in each group. The arrow indicates the final day of VRCTC-310 injection.

Toxicity

During the administration of VRCTC-310, dose-dependent toxicity at the injection site was observed. Toxic effects in mice were evidenced by varying degrees of muscle wasting, limping, and discrete areas of edema that disappeared once treatment was suspended. Such toxic effects appeared in the majority, but not all, of the treated animals.

Antitumor efficacy

As Fig. 3 (study I) shows, both the escalating low-dose and the fixed-dose schedules of VRCTC-310 produced antitumor responses against Lewis lung carcinoma. The escalating low-dose and the fixed-dose schedules produced a 44% and 48% decrease, respectively, in tumor volume on day 24 of VRCTC-310 therapy. The most effective therapy was the escalating high-dose regimen which resulted in an 83% decrease in tumor volume of treated mice when compared to the untreated control animals on day 20, one day after the final VRCTC-310 injection. One mouse from the treated group had no palpable tumor on day 24 and was permitted to live for a total of 60 days after tumor implant. A post 60-day autopsy indicated no evidence of disease. Data from the escalating high-dose group suggest that a higher dose of VRCTC-310 at the initiation of tumor therapy was more effective than lower doses in either fixed or escalating schedules.

In study II, the longer pretreatment with CT that mice were subjected to enabled an even higher initial dose of VRCTC-310 to be used for antitumor treatment than that used in study I. Inducing CT tolerance allowed us to start with a daily dose of 4.5 mg/kg VRCTC-310, which was progressively escalated to 6.5 mg/kg/day by the end of treatment on day 25. Mice received 4.5 mg/kg/day on days 1–12 and 5.0, 5.5, 6.0 and 6.5 mg/kg/day on days 13–15, 16–19, 20–22, and 23–25, respectively. The control group had to be sacrificed on day 23 because of excessive tumor volume whereas mice in the treated group were sacrificed on day 26. Figure 4 presents data on tumor-growth inhibition. In this experiment there was an 80% decrease in tumor volume in the treated group on day 22. One mouse in this group died of apparent toxicity on day 7, but there was no evidence of tumor at autopsy. The extent of tumor-growth inhibition obtained in study II was not significantly different from that observed in the escalating high-dose group in Study I, indicating that while inducing tolerance to the neu-

rotoxicity of the CT component is important, no gain was observed in the antitumor efficacy of VRCTC-310 when the starting treatment dose was increased from 1.8 to 4.5 mg/kg.

Discussion

While microbial- and plant-derived products have traditionally been thought of as sources of novel anticancer compounds, toxins of animal origin have received considerably less attention. The recent discoveries of such marine animal-derived products as bryostatin and dolastatin-10, however, represent novel compounds with interesting cellular targets (*i.e.*, protein kinase C and tubulin, respectively) [20,21], suggesting that other animal toxins or components derived from them may be useful anticancer agents as well. Early studies by Braganca *et al.* [3], and more recently by Kaneda *et al.* [4], showed selective toxicity by purified snake venom products toward neoplastic as opposed to normal cells in culture. Studies performed in collaboration with the National Cancer Institute demonstrated that the venom-derived product VRCTC-310 exhibited a unique pattern of cytotoxicity against CNS and melanoma and to some extent the lung tumor cell lines. This pattern of activity was submitted to COMPARE analysis and was found to be unique in that it was unlike that exhibited by any other agent previously run through this novel *in vitro* cell line panel.

While the specific mechanism(s) of cytotoxicity of VRCTC-310 are not fully understood, both components of VRCTC-310 (CT and CD) are known to be membrane-active substances. The cytotoxicity of CT requires PLA_2 activity [6] and the dissociation of its two subunits [6,22]. It has been suggested that it is in the targeting of PLA_2 enzymes to specific tissues or cells by which unique toxicities are produced [23]. Such target cells may possess receptor or acceptor sites that are recognized by a specific position of the enzyme [24]. This determines the binding of the PLA_2 to the cell membrane [23,24] and the subsequent phospholipid hydrolysis that results in cytotoxicity [6].

The contribution of the CD component to VRCTC-310 involves potentiation of CT cytotoxicity [33] and reduction of its neurotoxicity. Although specific interactions of CD with individual protein components of the cell membrane have been suggested, CD is generally thought to perturb cell membranes in a general manner by insertion into the lipid phase [25]. Synergism between CD and PLA_2s as demonstrated by enhanced cell lysis [26,27] is thought to result from CD-mediated action that alters the membrane structure and facilitates phospholipid hydrolysis mediated by the enzyme [28]. *In vitro*, CD has proved to selectively lyse transformed rather than normal cells [4], and be cytotoxic to various tumor cells, however, it has not displayed antitumor activity *in vivo* [34]. *In vivo* the CD component of VRCTC-310 displays certain pharmacologic interactions with the CT component, such as an increase in CT cytotoxicity and a decrease in CT neurotoxicity. It will be of considerable interest, therefore, to discover the specific membrane and/or cellular target sites associated with binding of VRCTC-310 to malignant cells *in vitro* and *in vivo* and the specific molecular role CD is performing in the observed synergism with CT (*e.g.*, whether as a chaperone molecule or otherwise).

While the cytolytic and cytotoxic properties of many animal venoms have been well described [1,2], the general toxicity associated with systemic administration of these agents has made *in vivo* evaluation of pharmacologic properties other than their intrinsic toxicities very difficult. The neurotoxicity associated with administration of toxins such as CT and VRCTC-310, which contains CT, can be largely prevented by inducing tolerance in animals. As this and other reports [7] show, mice can be made tolerant to the neurotoxicity associated with the administration of VRCTC-310 by being pretreated with daily escalating doses of other CT or VRCTC-310 itself. While the mechanism of tolerance induction is unclear, it is mechanistically independent from the development of antibodies to this venom component [7].

The results show that the induction of tolerance prior to treatment did not significantly affect tumor growth in control animals (Figs. 3 and 4). On the other hand, administering high doses of VRCTC-

310 to pretreated animals resulted in a higher degree of tumor-growth inhibition than low-dose treatments did in nonpretreated mice, where doses were more limited because of higher toxicity. Thus, the decrease in neurotoxicity caused by induction of tolerance does not result in decreased antitumor efficacy of VRCTC-310. The increased extent of growth inhibition observed in pretreated animals was probably related to the administration of higher doses of VRCTC-310. Therefore, neurotoxicity appears to be clearly separated from the antitumor effect and enables VRCTC-310 to have a useful therapeutic index. Optimization of the therapeutic index was explored in study II by prolonging the pretreatment and thus allowing a higher initial dosing after tumor implantation. The antitumor response observed in study II, however, was not different from that in study I, suggesting that after a certain dose-level of VRCTC-310 is reached, further increase do not contribute to added antitumor efficacy.

In addition to Lewis lung carcinoma, VRCTC-310 has been shown to possess antitumor efficacy against the MX-1 mammary carcinoma and the Walker 256 carcinosarcoma tumors (Vidal JC, Viskatis LJ, Etcheverry MA, unpublished observations). VRCTC-310 does not appear to be active against P388 murine leukemia, thus, suggesting that there may be specificity for individual types of solid tumors.

The local toxicity observed at the injection site could be attributed to properties of the VRCTC-310 components and the i.m. dose schedule. CT is myotoxic [29], and the evolution and reversibility of the muscle lesions have been described [30]. At high doses, CD produces muscle contracture [31] by depolarization of the cell membrane [16] and local inflammatory edema [32]. Although preliminary studies have so far shown that daily doses of VRCTC-310 are superior to other drug schedules, further testing of optimal dosing and scheduling and detailed pharmacokinetic studies of the individual components are needed. Such studies are currently in progress.

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