

Potential of anticancer-drug cytotoxicity by sea anemone pore-forming proteins in human glioblastoma cells

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The search for new drugs and treatment approaches is of particular importance for glioblastomas (GBMs), as with other types of malignant gliomas, as they are lethal without the available medical care. Current anticancer cocktails have failed to prolong survival beyond 1 year, in part owing to the natural resistance of GBM cells and to the toxic side effects of the available drugs. In many organisms, cell death can be induced by cytolytic proteins, which are proteins that can form pores in biological membranes. Perhaps by facilitating drugs to enter into the cytosol, cytolytic proteins might be used to increase the efficacy of conventional anticancer agents. Here, the cytotoxicity of two sea anemone pore-forming cytolytic proteins, toxin Bc2, and equitoxin (EqTx-II) were investigated. Toxin Bc2 and EqTx-II were cytotoxic against human U87 and A172 GBM cell lines either wild type or p53 mutant, a tumor suppressor frequently mutated in malignant gliomas. Moreover, noncytotoxic concentrations of Bc2 or EqTx-II potentiated the cytotoxicity induced by low dose concentrations of all classical chemotherapeutic agents tested: cytosine arabinoside, doxorubicin, and vincristine. In comparison with the cytotoxicity induced by each of these classical anticancer drugs alone, 10–300-fold less of the therapeutic

drug was needed when combined with the cytolytic proteins. These results are promising, since lower concentrations of chemotherapeutic drugs could reduce the adverse effects of chemotherapy. *Anti-Cancer Drugs* 19:517–525 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Glioblastoma (GBM), the most common subtype of primary brain tumors, is a malignant glioma characterized by a high proliferative index, aggressive invasiveness, and short patient survival [1]. GBMs present one of the greatest challenges in oncology. Although new treatment paradigms have had significant impact on the outcomes of many other cancer types, the efficacy of new agents to chemotherapy against malignant glioma has shown little improvement in decades [2]. Several trials have reported that raising the dose of currently available chemotherapeutic drugs does not get translated into a survival advantage, and it increases the occurrence of serious adverse effects in individuals under treatment [3]. Recently, there has been some progress in the analysis of cellular and molecular mechanisms of the resistance phenotype of brain tumors, giving new perspectives for cancer chemotherapy [3]. Attempts have been made to find new effective antitumor compounds or approaches against gliomas, such as the local delivery of chemother-

apeutic agents, targeted toxins, and gene therapy [4–7]. Despite the fact that pore-forming proteins have been found to be efficient against carcinomas [8–11], little effort has been aimed at using these toxins as a component for killing target cells.

Proteins and peptides with the capacity to increase membrane permeability are produced by a large number of organisms, and are one of Nature's most potent biological weapons [12]. One of the most common mechanisms behind the action of these toxins, referred as cytolytic proteins, is the formation of pores on the targeted cell membrane, an effect that can be cytotoxic and cytolytic [12]. An essential feature of their toxicity is the remarkable property that cytolytic proteins can exist either in a stable water-soluble state or as an integral membrane pore [12]. These cytolytic toxins can cause cell death either alone or in combination with antibodies or other molecules targeted against cancer cells [12]. For example, the diphtheria toxin fused with interleukin 2 is currently being evaluated in phase I/II clinical studies for the treatment of hematopoietic malignancies [10].

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In this study, we investigated the cytotoxicity of two sea anemone cytolytins, toxin Bc2 and equinatoxin-II (EqTx-II), against malignant gliomas. Toxin Bc2, which we isolated from the Brazilian sea anemone *Bunodosoma caissarum* [13], showed the ability to induce neurotransmitter release in nerve terminal preparations [13] and in neuroendocrine cells [14]. This effect resulted from the fact that toxin Bc2 increases the intracellular Ca^{2+} concentration [14], a preliminary step to initiate neurotransmitter release. The cytolytin EqTx-II, isolated from the sea anemone *Actinia equina* [15,16] has been sequenced, cloned, and expressed in *Escherichia coli* [15]. EqTx-II has the capacity to produce pores in natural and artificial lipid membranes by the association of three or four monomers [17]. Sea anemone cytolytins attack target cells by inserting themselves into the cell membrane, usually forming oligomeric transmembrane pores with a diameter of about 1–2 nm [17,18]. The toxic effects are caused by allowing the free flow of electrolytes and other small molecules (below 960 Da) across the membrane [16–18]. Alternatively, cytolytins might be used for cytosolic delivery of toxic peptides [4,19] or nucleic acids [20], and to increase the efficacy of conventional chemotherapeutic drugs facilitating their access into the cell. In this study, to potentiate the cell death of human GBMs, we used a novel strategy, the use of cytolytins toxin Bc2 and EqTx-II in a low concentration combined with three classical chemotherapeutic agents: Ara-C, doxorubicin, and vincristine.

Materials and methods

Chemicals and reagents

MTT was purchased from Sigma Chemical Co (St Louis, Missouri, USA). Cytosine arabinoside (Ara-C; Baxter, São Paulo, Brazil), doxorubicin (Bergamo Ltd., São Paulo, Brazil), and vincristine (Interlab Farmacêutica Ltd., São Paulo, Brazil) were dissolved in dimethyl sulfoxide, and the final concentrations of dimethyl sulfoxide in the culture medium did not exceed 0.1%. All other reagents were of analytical grade.

Preparation of toxin Bc2 and recombinant equinatoxin-II

Toxin Bc2 was isolated from the sea anemone *B. caissarum* as previously described [13]. EqTx-II was obtained according to Anderluh *et al.* [15]. Briefly, it was expressed from a T7-based expression vector in the *E. coli* BL21 (DE3) strain, and the recombinant protein was purified from bacterial supernatants as described [15]. Toxins Bc2 and EqTx-II were purified to homogeneity. The cytolytic activity of EqTx-II and toxin Bc2 was determined after incubation with a 5% sheep erythrocyte suspension [13]. The protein concentration was determined by the bicinchoninic acid method. For the cytotoxicity assay, toxin Bc2 and EqTx-II were dissolved in Dulbecco's minimum essential medium (DMEM).

Human glioblastoma cell lines

The human GBM cell lines U87 and A172 (ATCC) were grown in the medium consisting of DMEM and nutrient mixture F12 (DMEM-F12), enriched with glucose (33 mmol/l), glutamine (2 mmol/l), sodium bicarbonate (3 mmol/l) and nutrient mixture F12 supplemented with 10% fetal bovine serum (FBS) [21]. The cells were cultured in a 37°C incubator with 5% CO_2 .

Astrocyte primary cultures

Astrocyte primary cultures were prepared from cerebral cortices of newborn (P0) rats as previously described [22]. Briefly, after the rats had been decapitated, brain structures were removed and the meninges carefully stripped off. Tissues were washed in phosphate buffered saline (PBS)–0.6% glucose and dissociated into single cells in DMEM-F12. Cells were seeded at a density of 2.0×10^5 cells/cm² either on glass coverslips or on 25 cm² culture flasks previously coated with polyornithine (1.5 µg/ml), in DMEM-F12 supplemented with 10% FBS. Cultures were maintained at 37°C in a humidified 5% CO_2 chamber. Culture medium was changed for the first time at 24 h after plating, and then at 3 days intervals. After confluence was reached (10 days), cell cultures were washed and treated in serum-free DMEM-F12 medium with toxin Bc2 or EqTx-II for 24 h at 37°C.

Cytotoxicity assays

U87 and A172 cells were plated in 96-well culture plates (10^4 cells/well) in 10% FBS DMEM-F12 medium and cultured for 24 h for reaching confluence. After this period, cells were washed and treated in serum-free DMEM-F12 medium with toxin Bc2 or EqTx-II and/or chemotherapeutic drugs: Ara-C, doxorubicin or vincristine for 24 h at 37°C. Viable cells were quantified by the MTT tetrazolium dye colorimetric assay for mitochondrial dehydrogenase, as previously described [23]. In some experiments, the MTT stained cultures were photographed under the phase contrast microscope.

For the nuclear morphology analysis, U87 cells were plated on sterilized glass coverslips in 24-well plates with 10% FBS DMEM-F12 medium for 24 h. After that, cultures were washed and treated in serum-free DMEM-F12 medium with EqTx-II and/or vincristine for 24 h at 37°C. Cells were fixed with 4% paraformaldehyde in PBS for 20 min, and stained with 4',6-diamidino-2-phenylindole (DAPI) in PBS. Next, cells were photographed using appropriate ultraviolet filters to examine DAPI fluorescence staining under the microscope. At least 200 random cells were quantified in each condition.

Statistical analysis

The results are presented as the mean \pm SEM of independent experiments, except the IC_{30} values (i.e. the concentration required for 30% cytotoxicity), which are reported as geometric means. The IC_{30} value was

determined by linear regression from individual experiments using GraphPad software (GraphPad software Inc., San Diego, California, USA). Statistical significance was determined by unpaired Student's *t*-test or one-way analysis of variance, and $P < 0.05$ was considered to be significant.

Results

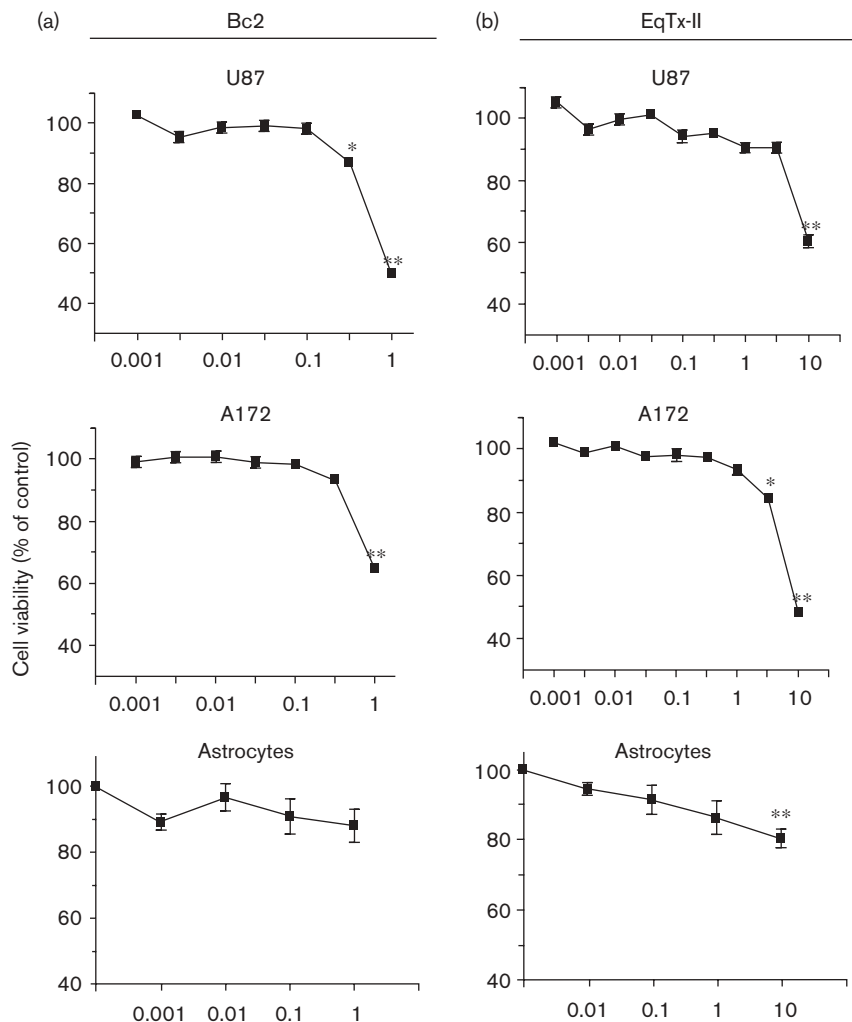
Toxin Bc2 and equinatoxin-II decrease viability of glioblastomas cells

The effect of sea anemone cytolyins on human GBM cells viability was investigated. U87 (p53 wild type) or A172 (p53 mutant) cell lines were cultured in the presence of increasing concentrations of toxin Bc2

(0.001–1 $\mu\text{g/ml}$) or EqTx-II (0.001–10 $\mu\text{g/ml}$) for 24 h, and the cellular viability was evaluated by the MTT test. Toxin Bc2 did not exert cytotoxicity under 0.1 $\mu\text{g/ml}$ (Fig. 1a). At 1 $\mu\text{g/ml}$, however, toxin Bc2 significantly reduced the U87 and A172 cellular viability to 50 ± 2 and $65 \pm 2\%$, respectively. EqTx-II (10 $\mu\text{g/ml}$) decreased the U87 and A172 viability to 60 ± 2 and $48 \pm 1\%$, respectively (Fig. 1b). Therefore, these two GBM cell lines are sensitive to high dose of the two sea anemone cytolyins tested.

To determine the cytotoxicity induced by toxin Bc2 and EqTx-II in normal cells, we tested the cellular viability of normal rat astrocytes after treatment with the sea anemone cytolyins (24 h). As shown in Fig. 1a (lower

Fig. 1



Dose-response effect of toxin Bc2 and equinatoxin (EqTx-II) upon glioblastomas (GBMs) and astrocytes cell death. Graphics show the percentage of cellular viability, determined by the MTT method, of U87 (upper panels) and A172 human GBM cells (middle panels) and astrocytes primary cultures of newborn (P0) rats (lower panels) 24 h after treatment with the indicated doses of toxin Bc2 (a) or EqTx-II (b). The cell viability is expressed as percentage of the control (control = Dulbecco's minimum essential medium, 100%). Data are expressed as the mean \pm SEM of three independent experiments (done in triplicate). * $P < 0.05$ and ** $P < 0.01$ (one-way analysis of variance, Dunnett's post test) in comparison with the untreated control values (100%).

panel), toxin Bc2 did not significantly reduce the astrocyte viability when compared with untreated values, whereas EqTx-II at 10 µg/ml decreased viability to $80.3 \pm 2.7\%$ (Fig. 1b). Moreover, toxin Bc2 (1 µg/ml) and EqTx-II (10 µg/ml) were significantly less toxic to astrocytes than to glioma cells (U87 and A172), when we compared the cytotoxicity effect induced by the same concentrations ($P < 0.05$, Student's *t*-test, unpaired).

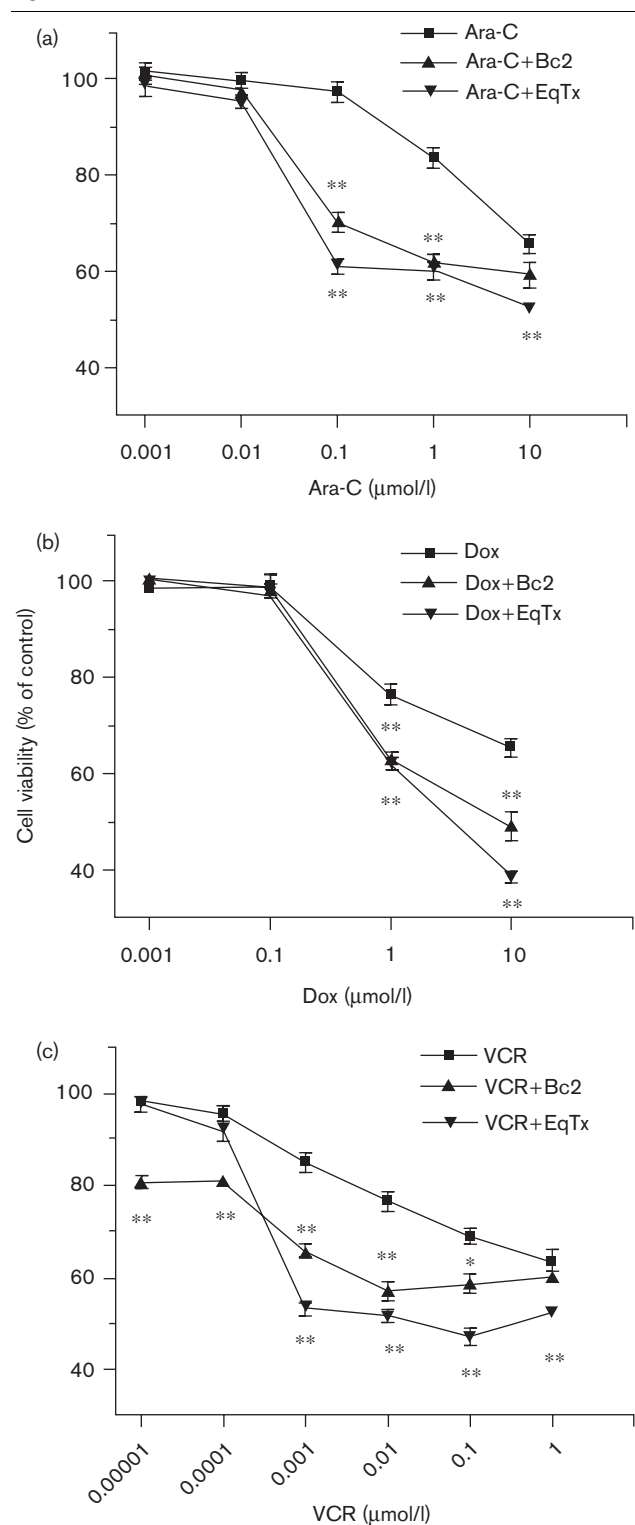
Chemotherapeutic agent-induced cell death is enhanced by cytotoxins

The three chemotherapeutic agents tested (Ara-C, doxorubicin, and vincristine) induce cell death in many tumor lines [24]. Here, we examined whether noncytotoxic concentrations of toxin Bc2 (0.1 µg/ml) or EqTx-II (0.3 µg/ml) might enhance the cell death induced by the chemotherapeutic agents Ara-C, doxorubicin, and vincristine on GBM cells. In U87 cells, the treatment with increasing concentrations of Ara-C caused a dose-dependent induction of cell death (Fig. 2a). Ara-C at 8.4 µmol/l induced 30% cytotoxicity (IC_{30} , Table 1). In combination with 0.1 µg/ml of toxin Bc2, however, only 0.6 µmol/l of Ara-C was required to reach IC_{30} . Moreover, merely 0.5 µmol/l of Ara-C was required to induce 30% cytotoxicity if combined with 0.3 µg/ml of EqTx-II. Therefore, the IC_{30} ratio was increased 14 folds for Ara-C/Ara-C plus Bc2 and 17 folds for Ara-C/Ara-C plus EqTx-II. When pore-forming cytotoxins were tested in combination with doxorubicin, the toxin Bc2 and EqTx-II also decreased the IC_{30} of doxorubicin in 9.6 folds (Fig. 2b, Table 1). The IC_{30} values were 7.7 µmol/l for doxorubicin treatment alone, and 0.8 µmol/l of doxorubicin when combined with either toxin Bc2 or EqTx-II (Fig. 2b).

Vincristine was the next chemotherapeutic agent to be tested. Toxin Bc2 (0.1 µg/ml) and EqTx-II (0.3 µg/ml) significantly increased the cytotoxicity induced by vincristine in all the concentration range tested (1 to 0.00001 µmol/l) in U87 cells (Fig. 2c). The IC_{30} for vincristine was 0.3 µmol/l, compared with 0.002 and 0.001 µmol/l for the combination of vincristine plus toxin Bc2 or vincristine plus EqTx-II respectively. This represents an increase in the IC_{30} ratio of 150-fold for vincristine/vincristine plus Bc2, and of 300-fold for

vincristine/ vincristine plus EqTx-II (Table 1). On A172 cells, noncytotoxic concentrations of Bc2 and EqTx-II also potentiated the effect of low dose anticancer drugs (Table 2).

Fig. 2



Enhancement of chemotherapeutic-induced cell death by pore-forming cytotoxins in U87 cells. U87 cells were treated (24 h) with increasing concentrations of anticancer drugs alone or in combination with noncytotoxic concentrations of toxin Bc2 (0.1 µg/ml) or equinatoxin-II (EqTx-II) (0.3 µg/ml). Cell viability was measured by the MTT method. Toxin Bc2 and EqTx-II augmented the cell death induced by cytosine arabinoside (Ara-C) (a), doxorubicin (b), and vincristine (c). Data are expressed as the mean \pm SEM of three independent experiments (done in triplicate). * $P < 0.05$ and ** $P < 0.01$ (Student's *t*-test, unpaired) when comparing the anticancer drug versus the combination of anticancer drugs plus cytotoxins. Dox, doxorubicin; VCR, vincristine.

Table 1 Cytotoxicity (IC₃₀) of chemotherapeutic drugs alone or combined with toxin Bc2 or EqTx-II against U87 cells

Compound	IC ₃₀ (μmol/l)	+ Bc2		+ EqTx-II	
		IC ₃₀	Ratio ^a	IC ₃₀	Ratio ^a
–	–	0.7 μg/ml	–	8.5 μg/ml	–
Ara-C	8.4	0.6 μmol/l	14	0.5 μmol/l	17
Doxorubicin	7.7	0.8 μmol/l	9.6	0.8 μmol/l	9.6
Vincristine	0.3	0.002 μmol/l	150	0.001 μmol/l	300

IC₃₀ values (μg/ml or μmol/l) were obtained from geometric means of three experiments carried out in triplicates.

Ara-C, cytosine arabinoside; EqTx-II, equinatoxin-II.

^aIC₃₀ ratios (chemotherapeutic drug/chemotherapeutic drug + toxin Bc2 or EqTx-II) were then calculated from the IC₃₀ values obtained.

Table 2 Cell viability (% of control) of chemotherapeutic drugs alone or combined with toxin Bc2 or EqTx-II against U87 and A172 cells

	U87			A172		
	–	+ Bc2	+ EqTx-II	–	+ Bc2	+ EqTx-II
–	–	98 ± 1.7	95 ± 1.5	–	98 ± 1.3	97 ± 1
Ara-C	84 ± 1	62 ± 2**	60 ± 1**	80 ± 3	65 ± 2**	73 ± 1*
1 μmol/l						
Dox	76 ± 2	63 ± 2**	61 ± 1**	75 ± 2	68 ± 3*	62 ± 2**
1 μmol/l						
Vincristine	85 ± 1	66 ± 1**	53 ± 2**	84 ± 3	75 ± 1**	73 ± 3**
0.001 μmol/l						

The cell viability is expressed as percentage of the control (control=DMEM, Dulbecco's minimum essential medium, 100%) of toxin Bc2 (0.1 μg/ml)- or EqTx-II (0.3 μg/ml)-treated cells using the MTT method. Data are expressed as the mean ± SEM of three independent experiments, done in triplicate.

Ara-C, cytosine arabinoside; Dox, doxorubicin; EqTx-II, equinatoxin-II.

P* < 0.05 and *P* < 0.01 (Student's *t*-test, unpaired) when comparing cellular viability obtained using the combinatory treatment with the respective chemotherapeutic treatment alone.

Denatured pore-forming proteins did not enhance chemotherapeutic agent-induced cell death

To confirm whether the enhanced cytotoxicity effect induced by toxin Bc2 and EqTx-II was owing to the isolated cytolysins proteins, a cell viability assay was carried out with combinations of Ara-C, doxorubicin, or vincristine and denatured (incubated at 100°C for 5 min) toxin Bc2 (0.1 μg/ml) or EqTx-II (0.3 μg/ml). When the cells were treated with a combination of 0.1 μmol/l Ara-C and toxin Bc2 or EqTx-II, the cell death was decreased by about 30 and 40%, respectively, compared with untreated controls. The treatment with the same concentration of Ara-C and boiled toxin Bc2 or EqTx-II, however, had no effect on cell viability compared with the untreated controls (Fig. 3a). A similar result was obtained when this assay was carried out with 1 μmol/l doxorubicin and denatured Bc2 or EqTx-II proteins (Fig. 3b). Heated cytolytic proteins also exerted no synergistic effect on the cell death induced by 0.001 μmol/l vincristine (Fig. 3c). Therefore, these results suggest that the potentiation and synergism

of cytotoxicity induced by the combinatory cocktails were because of the active cytolysins, known to be sensitive to heat inactivation [25].

Vincristine-induced cell death is enhanced by high concentrations of toxin Bc2

We also tested whether higher concentrations of sea anemone cytolysin combined with low dose of a chemotherapeutic drug would show increased cytotoxicity compared with the cell death observed after each treatment alone. We chose concentrations of toxin Bc2 that were cytotoxic on U87 cells (0.5–1 μg/ml), in order to determine whether this treatment combined with vincristine (0.001 μmol/l) would still show increased cell death compared with cytotoxicity observed following the individual treatment. Under control conditions (set as 100% viability), U87 cells showed fusiform morphology with processes characteristic of these cells (data not shown). After 24 h of drug exposure, vincristine (0.001 μmol/l) alone exerted a small effect on the cellular viability (84.1 ± 2.2%; Fig. 4) and the U87 cells became rounded without processes. Toxin Bc2 (1 μg/ml) alone reduced the number of viable cells to 56.5 ± 2% (Fig. 4) and did not seem to alter the cellular morphology (data not shown). When U87 cells were incubated simultaneously with Bc2 (1 μg/ml) and vincristine (0.001 μmol/l), the cell viability significantly decreased to 23 ± 4.6% (Fig. 4), with increased amount of nonviable and rounded cells without processes. Therefore, low doses of vincristine or associated with Bc2 promoted a morphological effect on the cells, which is expected for an alkaloid that disrupts the cellular cytoskeleton [26–28]. Moreover, these results showed that the combined treatment with cytotoxic concentration of toxin Bc2 were able to further enhance cell death in the presence of a classic chemotherapeutic drug.

Pore-forming protein enhances vincristine-induced mitotic arrest

To investigate whether low doses of cytolysins might have enhanced the intracellular level of chemotherapeutic drugs, we examined vincristine nuclei effect, either alone or in combination with cytolysins, by DAPI nuclear staining. Vincristine is a microtubule-disturbing agent known to arrest cells with condensed chromosomes at the M phase of the cell cycle [26]. Therefore, the incubation with increasing concentrations of vincristine is followed by rising percentage of mitotic-like figures in treated cultures [27]. The percentage of nuclei with M phase morphology in U87 cultures treated with 0.001 mmol/l of vincristine for 24 h was 17.2 ± 6.1%. Vincristine-induced mitotic arrest, however, increased to 33.2 ± 0.7% by simultaneous incubation with sublethal doses of the cytolysin EqTx-II (0.3 μg/ml). The treatment with EqTx-II (0.3 μg/ml) alone has no effect on the percentage of M phase cells

(Fig. 5). These results showed that sea anemone cytolytins enhanced the vincristine action in human GBM cells.

Fig. 3

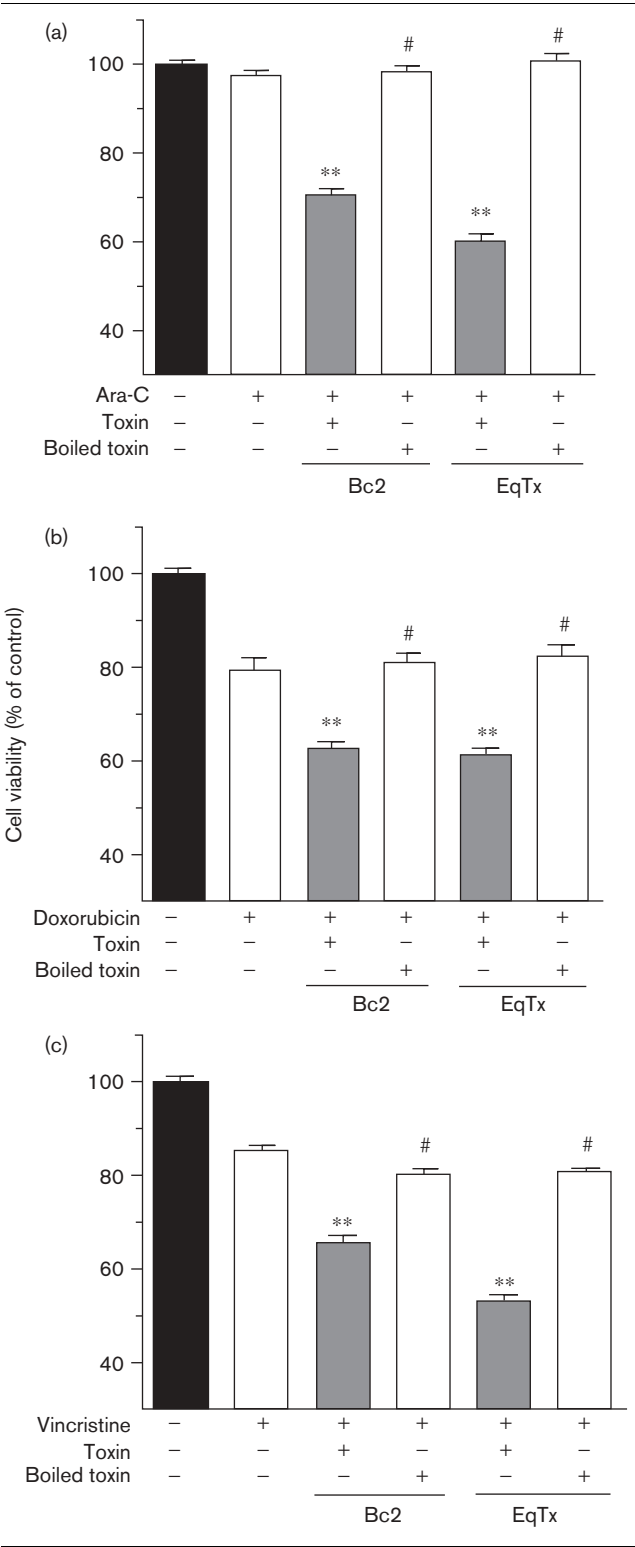
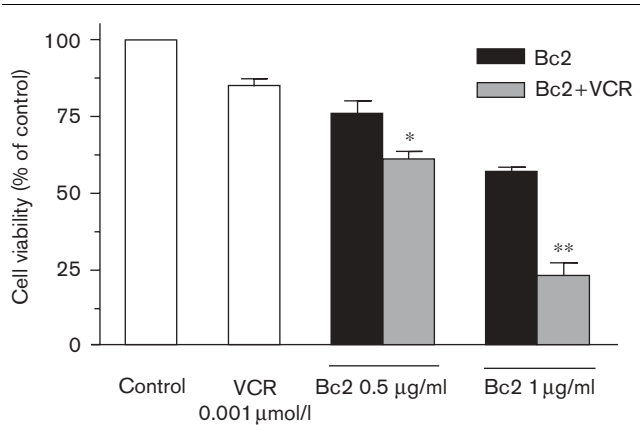


Fig. 4



Enhancement of the vincristine-induced cell death by high concentrations of toxin Bc2. Histogram shows the percentage of U87 cellular viability, as measured by the MTT method, with the indicated treatments. U87 cells were kept in control conditions, or treated with vincristine (VCR) (0.001 µmol/l), or with VCR (0.001 µmol/l) in combination with toxin Bc2 (0.5 or 1 µg/ml) for 24 h. Data are expressed as the mean \pm SEM of three independent experiments (done in triplicate). * $P < 0.05$ and ** $P < 0.01$ (Student's *t*-test, unpaired) when comparing the Bc2 treatment versus the combination Bc2 plus VCR.

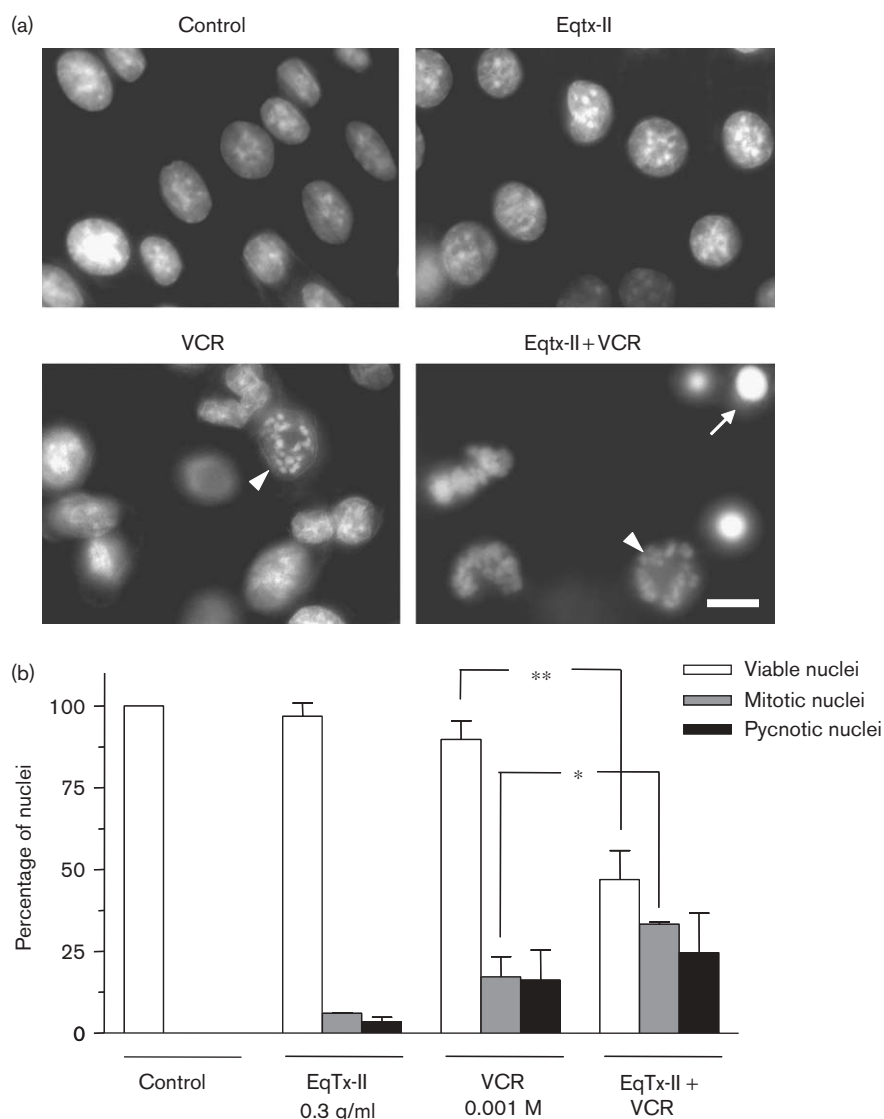
Discussion

The potential use of pore-forming toxins to generate a novel class of anticancer drugs has not been extensively explored. In this study, we demonstrated that two sea anemone cytolytins, toxin Bc2 and EqTx-II, could induce cytotoxicity on U87 and A172 human GBM cells. Moreover, noncytotoxic concentrations of cytolytins were able to enhance the cytotoxicity induced by low dose of anticancer agents on both U87, a p53 wild type, and A172, a p53 mutant [29] GBM cell lines.

Lavie and Liscovitch [30] showed that cancer cells have marked changes in membrane lipid composition. Most notably, elevated levels of cholesterol, glycosphingolipids, and sphingomyelin have been reported [31]. As sea anemone pore-forming cytolytins, such as toxin Bc2 and EqTx-II, require high levels of sphingomyelin or cholesterol for membrane binding and permeabilization

Denatured pore-forming proteins exerted no effect on cell death induced by anticancer drugs. Cell viability was determined by the MTT assay of U87 cultures kept in control conditions or after the treatment with chemotherapeutic agents alone or in combination with boiled or unboiled proteins. Heating toxin Bc2 (0.1 µg/ml) and equinatoxin-II (EqTx-II) (0.3 µg/ml) fail to enhance the cell death induced by 0.1 µmol/l cytosine arabinoside (Ara-C) (a), 1 µmol/l doxorubicin (b) or 0.001 µmol/l (1 nmol/l) vincristine (c). Data are expressed as the mean \pm SEM of three independent experiments (done in triplicate). ** $P < 0.01$ (Student's *t*-test, unpaired) when comparing the anticancer drug versus the combination of anticancer drugs plus unboiled cytolytins. # $P > 0.05$ (Student's *t*-test, unpaired) when comparing the anticancer drug versus anticancer drug plus boiled proteins.

Fig. 5



Pore-forming protein enhances vincristine (VCR)-induced mitotic arrest. Black and white fluorescent photomicrographs show nuclear 4',6-diamidino-2-phenylindole staining of U87 cultures. Cultures were either treated with VCR (0.001 μ M), equinatoxin-II (EqTx-II) (0.3 μ g/ml), VCR (0.001 μ M) in combination with EqTx-II (0.3 μ g/ml), or kept in control conditions for 24 h. Scale bar: 10 μ m (a). Arrowheads indicate nuclei consistent with M phase morphology. Arrow points to a pycnotic nucleus. Histogram shows the percentage of viable cells, pycnotic nuclei and mitotic figures (b). Data are expressed as the mean \pm SEM of three independent experiments. * P < 0.05 and ** P < 0.01 (Student's t -test, unpaired) when comparing vincristine versus the combination of VCR plus EqTx-II.

[16–18], these proteins might act more selectively against cancer cells. Accordingly, toxin Bc2 (1 μ g/ml) and EqTx-II (10 μ g/ml) were significantly more toxic to GBM cells, when compared with astrocytes in culture. In addition, we have previously demonstrated that nerve terminals (synaptosomes) incubated with toxin Bc2 (10 or 50 μ g/ml) did not show any significant leakage of the cytosolic marker lactate dehydrogenase, suggesting that the plasma membrane was not disrupted in neuronal cells [13].

In this study, we used a novel strategy: the use of pore-forming cytotoxins to potentiate the cytotoxicity induced

by low dose of anticancer drugs. We selected three anticancer agents with different mechanism of action: Ara-C (a DNA polymerase inhibitor), doxorubicin (a topoisomerase inhibitor), and vincristine (a microtubule-disturbing agent). Using the human malignant glioma cell lines U87 and A172, we examined the cytotoxicity induced by these chemotherapeutic agents alone and in combination with pore-forming proteins. The toxicity induced by the treatment of chemotherapeutic drugs alone was similar to previous study in various malignant glioma cell lines [24]. Furthermore, noncytotoxic concentrations of toxin Bc2 (0.1 μ g/ml) and EqTx-II

(0.3 µg/ml) increased the cytotoxic activity caused by all chemotherapeutic agents tested. These sea anemone cytolytins showed a prominent cytotoxic effect with the antimicrotubule agent vincristine: in U87 cells, the cytolytins enhanced up to 300 folds the cytotoxicity induced by vincristine. Altogether, we showed that low doses of chemotherapeutic drugs induced a more effective cytotoxicity when combined with pore-forming cytolytins.

The mechanism of potentiating cell death can be a direct consequence of increased drug delivery to the cytosol; this is particularly important for hydrophilic macromolecule anticancer agents, such as gelonin that has limited access to its target subcellular compartment [4]. Sea anemone cytolytin pores were estimated to be around 1–2 nm in diameter and permit the flow of molecules under 960 Da [16–18]. A recently published study reported the permeabilization of COS-7 cells to the fluorescent dye propidium iodide (668 Da), when the cells were incubated with the sea anemone cytolytin sticholysin-II (0.2–1 µg/ml) [30]. It should be noted that nearly all agents used to treat GBMs [24] have a molecular mass less than 960 Da, including all drugs used in this study (Ara-C: 243 Da, doxorubicin: 580 Da, and vincristine: 923 Da). Vincristine was the largest drug used in this study; however, it is the most hydrophobic anticancer compound among them. This hydrophobic property allows it to enter into the cell also by passive membrane diffusion [32]. The increased efficiency of vincristine uptake owing to low concentrations of pore-forming proteins might have increased vincristine intracellular levels to cause a synergistic effect.

The mechanism of inducing cell death mediated by cytolytins is subject of further investigation as cytolytins may also have intracellular targets. Several publications have pointed to p38 mitogen-activated protein kinase as an activation target in cells permeabilized by bacterial pore-forming toxins such as pneumolysin and streptolysin O [33–35]. It is worth mentioning that pore-forming proteins used here were cytotoxic regardless to multidrug resistance (data not shown) and p53 [29] status, a tumor suppressor frequently mutated in GBMs.

The development of peptide analogs of sea anemone cytolytins, such as that carried out recently [36] could allow their use in combination with chemotherapeutic agents for in-vivo studies. This opens the possibility for the pharmacological treatment of cancer with new synthetic peptides that mimic the pore-forming action, and potentiate the anticancer drug's effect. In addition, in an interesting study, Ellerby *et al.* [7] engineered a native-like pore-forming protein that shows antitumor activity in several animal models of cancer. *In vitro*, this engineered pore-forming protein shows no selectivity

toward normal or malignant cells, whereas its cytotoxic activity *in vivo* seems to be limited to tumors.

Cytolytins could also be used in therapies conjugated to antibodies or other molecules targeted against cancer cells [12]. For example, the cytolytin diphtheria toxin conjugated with transferrin was used in a phase I study with malignant central nervous system lesions, and established the safety, efficacy, and suggested dose limits. In addition, phase II and III trials are currently under way with promising preliminary results in high-grade gliomas [37].

It is imperative to find new drugs and alternative treatments for lethal cancers such as GBM; current chemotherapeutic cocktails fail to increase patient survival beyond 1 year, and usually lead to poor quality of life owing to their side effects [1]. For example, a reason to decrease the vincristine dosage is the presence of peripheral neuropathy, a common side effect. It is possible that a cocktail including a cytolytin would allow vincristine-induced cytotoxicity with minimal amount of this chemotherapeutic. In addition, cytolytin could also contribute to cell death by affecting direct targets in the cells, and not only by increasing cellular permeability to drugs [12,33–35]. Regardless of the mechanism of enhancing cytotoxicity, our findings suggest that the combination of pore-forming proteins with chemotherapeutic agents could increase the outcome of anticancer drugs.

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