

Inhibition of MAPK/ERK, PKC and CaMKII Signaling Blocks Cytolysin-induced Human Glioma Cell Death

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Abstract. *Background:* Cytolysins are pore-forming toxins that show anticancer activity by a mechanism hitherto poorly investigated. *Materials and Methods:* To investigate how cytolysins are cytotoxic to resistant cancer cells, proliferation and cell death were evaluated on U87 glioblastoma cells treated with toxin Bc2 or equinatoxin-II (EqTx-II). *Results:* Toxins Bc2 and EqTx-II decreased cell viability and increased lactate dehydrogenase (LDH) release in a concentration-dependent manner. Swollen, dead or dying cells were negative for TUNEL staining. The pre-treatment with inhibitors of mitogen-activated/extracellular regulated kinase (MEK1), protein kinase C (PKC) or Ca^{2+} /calmodulin-dependent kinase II (CaMKII) blocked the toxic effects of toxin Bc2 and EqTx-II, suggesting that calcium entry, activation of MEK1, PKC and CaMKII pathways are involved in the cytotoxicity induced by these cytolysins. *Conclusion:* Cytolysins were shown to be toxic to glioblastoma cells by activating several intracellular signaling pathways and resulting in necrosis-like cell death.

The potential use of pore-forming toxins to generate a novel class of anticancer drugs has been explored recently. The common mechanism of action of these toxins, also referred to as cytolysins, is the formation of pores on the targeted cell membrane that can be cytotoxic and cytolytic (1). Cytolysins either alone, or as hybrid proteins, fused to molecules, have

been used to target and kill cancer cells selectively (2, 3). These proteins are produced and released by bacteria and higher organisms (4), such as sea anemones (5). Equinatoxin II (EqTx-II), isolated from the sea anemone *Actinia equina* (2), has the capacity to produce pores in natural and artificial lipid membranes (6). Recently, EqTx-II and toxin Bc2, a cytolysin isolated from the sea anemone *Bunodosoma caissarum* (7), was shown to be a promising tool to selectively kill cancer cells including fibrosarcomas, breast carcinomas and glioblastomas (8, 9). Moreover, when given at non-toxic concentrations, EqTx-II enhanced up to 300-fold the cytotoxicity of the antimicrotubule agent vincristine in glioblastomas (8), showing that these cytolysins can also target cancer cells when used in combination with conventional chemotherapeutic drugs.

Glioblastomas are the most common subtype of gliomas and are considered one of the deadliest human cancers (10). Despite advances in cancer biology, the mean survival for patients with malignant gliomas remains less than approximately 14 months (11) due, in part, to its high resistance to currently used anticancer drugs (12). New efforts are focused on finding novel effective anticancer compounds, therapies such as gene therapy, local delivery of chemotherapeutics, targeted toxins and pore-forming proteins (12-15). It is also particularly important to understand the intracellular mechanism of cell death induced by these new anticancer compounds.

The mechanism of inducing cell death mediated by cytolysins is a subject of investigation as these pore-forming proteins may also have intracellular targets or activate signal transduction pathways to induce cell death (16). EqTx-II induces a Ca^{2+} influx in bovine lactotrophs (17) and in neuroblastoma cells (18); these Ca^{2+} and water influxes cause cell swelling (18). The increase in Ca^{2+} concentration can trigger one or more Ca^{2+} -dependent kinase cascades leading to survival or cell death. Several pathways and kinases are sensitive to changes in intracellular Ca^{2+} levels,

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including the mitogen-activated protein kinase/ extracellular-signal-regulated kinase (MAPK/ERK) pathway, cAMP dependent protein kinase A (PKA), Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), Ca^{2+} /phospholipid-dependent protein kinase (PKC), and phosphatidylinositol-3-kinase (PI3K) (19). Given that EqTx-II and toxin Bc2 increase the levels of Ca^{2+} , this paper aimed to investigate their mechanism of cytotoxicity in human malignant glioma cells, and some of the possible cell signaling pathways involved in their action, particularly the MAPK/ERK, PKC, PKA, CaMKII and PI3K pathways.

Materials and Methods

Isolation of toxin Bc2 and recombinant EqTx-II. Toxin Bc2 was isolated from the sea anemone *Bunodosoma caissarum* as described by Migueis *et al.* (7). EqTx-II was obtained according to Anderluh *et al.* (20). Toxin Bc2 and EqTx-II were purified to homogeneity, as observed in 12% SDS/PAGE gels and their cytolytic activity was determined after incubation with a 5% sheep erythrocyte suspension. Protein concentration was confirmed by the bicinchoninic acid method.

Cell culture and treatments. Human glioblastoma cell line U87 (ATCC) was grown in a medium consisting of Dulbecco's minimum essential medium (DMEM) and nutrient mixture F12 (DMEM-F12; Gibco/BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), as described by Faria *et al.* (21). For the viability assay, U87 cell monolayers were plated (10^4 cells/well) in 96-well culture plates and cultured in 10% FBS DMEM-F12 for 24 h. After this period, cells were washed and treated with toxin Bc2 or EqTx-II in a serum-free DMEM-F12 medium for 24 h at 37°C. PD98059 (10 μM), staurosporine (1 nM), KN-62 (10 μM), LY294002 (5 μM) (Sigma Chemical Co, USA) or H89 (5 μM ; Alomone Labs, Jerusalem, Israel) were added 10 min prior to the addition of toxin Bc2 or EqTx-II.

Cell viability and [H^3]-thymidine incorporation assay. Viable cells were quantified by the MTT colorimetric assay for mitochondrial dehydrogenase, as described by Gardner *et al.* (22). For [H^3]-thymidine incorporation analysis, cells were seeded at a density of 2×10^4 cells/well in 48-well culture plates and cultured for 24 h. After being incubated for 18 h with the indicated concentrations of toxin Bc2 or EqTx-II in serum-free DMEM-F12 medium, cells were pulsed with [H^3]-thymidine (2 $\mu\text{Ci}/\text{ml}$, Amersham Biosciences, Piscataway/NJ, USA) for an additional 6 h. Cells were washed with PBS, fixed in 10% trichloroacetate (TCA) and analysed for radioactivity in a liquid scintillometer.

A control group (no treatment) was set as 100% of [H^3]-thymidine incorporation.

Measurement of lactate dehydrogenase activity. Extracellular and intracellular lactate dehydrogenase (LDH) activity was measured using a Cytotoxicity Cell Death kit (Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer's instructions. Total LDH activity was defined as the sum of intracellular and extracellular LDH activity; released LDH was defined as the percentage of extracellular activity compared with total LDH activity.

TUNEL assay and cytochemistry cell labeling. To investigate the type of cell death, detection of DNA fragments *in situ* was performed by the TUNEL technique (Terminal Deoxynucleotide Transferase dUTP Nick End Labeling, TUNEL Assay Kit, Molecular Probes, Eugene, OR, USA) following the manufacturer's instructions. Cells were then nuclear stained with 4',6-diamidino-2-phenylindole (DAPI) in PBS. For cell morphologic analysis, U87 cells were fixed and stained using the Panoptic haematological labeling method according to the manufacturer's instructions (Laborclin, Curitiba, Brazil). After staining and air-drying, glass coverslips with toxin-treated or control cells were mounted with Entellan® (Merck, Darmstadt, Germany).

Statistical analysis. The results are presented as the mean \pm standard error of the mean (S.E.M). Statistical significance was determined by one-way analysis of variance (ANOVA). $P < 0.05$ was considered to be statistically significant.

Results

Toxins Bc2 and EqTx-II decreased U87 [H^3]-thymidine incorporation and viability. In order to investigate whether cytotoxins would influence the cellular proliferation, U87 cell monolayers were treated with increasing concentrations of toxins Bc2 (0.001-1 $\mu\text{g}/\text{ml}$) and EqTx-II (0.001-10 $\mu\text{g}/\text{ml}$) for 18 h. For labeling cells at the S-phase of the cell cycle, monolayers were then pulse-labeled with [H^3]-thymidine for additional 6 h. Both sea anemone cytotoxins decreased the amount of [H^3]-thymidine labeled cells at 1 $\mu\text{g}/\text{ml}$ (Figures 1A, B).

Next, the viability of U87 cells was investigated under cytotoxin incubation for 1 h, 3 h, 6 h and 24 h by measuring the mitochondrial dehydrogenase activity (MTT assay) (Figures 1C, D). Treatment with 0.01 $\mu\text{g}/\text{ml}$ toxin Bc2 or 0.1 $\mu\text{g}/\text{ml}$ EqTx-II was not cytotoxic to U87 cells at any time point investigated. However, 1 $\mu\text{g}/\text{ml}$ of toxin Bc2 and 10 $\mu\text{g}/\text{ml}$ EqTx-II decreased viability in U87 cells to about 50% after 6 h. In addition, 10 $\mu\text{g}/\text{ml}$ EqTx-II decreased cellular viability significantly after as early as 3 h of incubation.

Toxin Bc2 and EqTx-II induced cell death in U87 human glioma cells. Although 1 $\mu\text{g}/\text{ml}$ toxin Bc2 and 10 $\mu\text{g}/\text{ml}$ EqTx-II decreased the U87 cell viability, it was not clear which mechanism of cell death was triggered by cytotoxins. Since LDH release is used as an indicator of necrosis (23), we performed an LDH release assay in U87 cells treated with increasing concentrations of toxin Bc2 (0.001-1 $\mu\text{g}/\text{ml}$) or EqTx-II (0.001-10 $\mu\text{g}/\text{ml}$). Toxin Bc2 did not induce LDH release on U87 cells at concentrations less than 0.1 $\mu\text{g}/\text{ml}$; however, at 1 $\mu\text{g}/\text{ml}$, toxin Bc2 increased the LDH release significantly to nearly 50% (Figure 2A). EqTx-II at 0.001 $\mu\text{g}/\text{ml}$ produced no alterations in LDH release; however, the LDH release was enhanced to 25% at 0.01 $\mu\text{g}/\text{ml}$, and 40% at doses above 0.1 $\mu\text{g}/\text{ml}$ EqTx-II (Figure 2B).

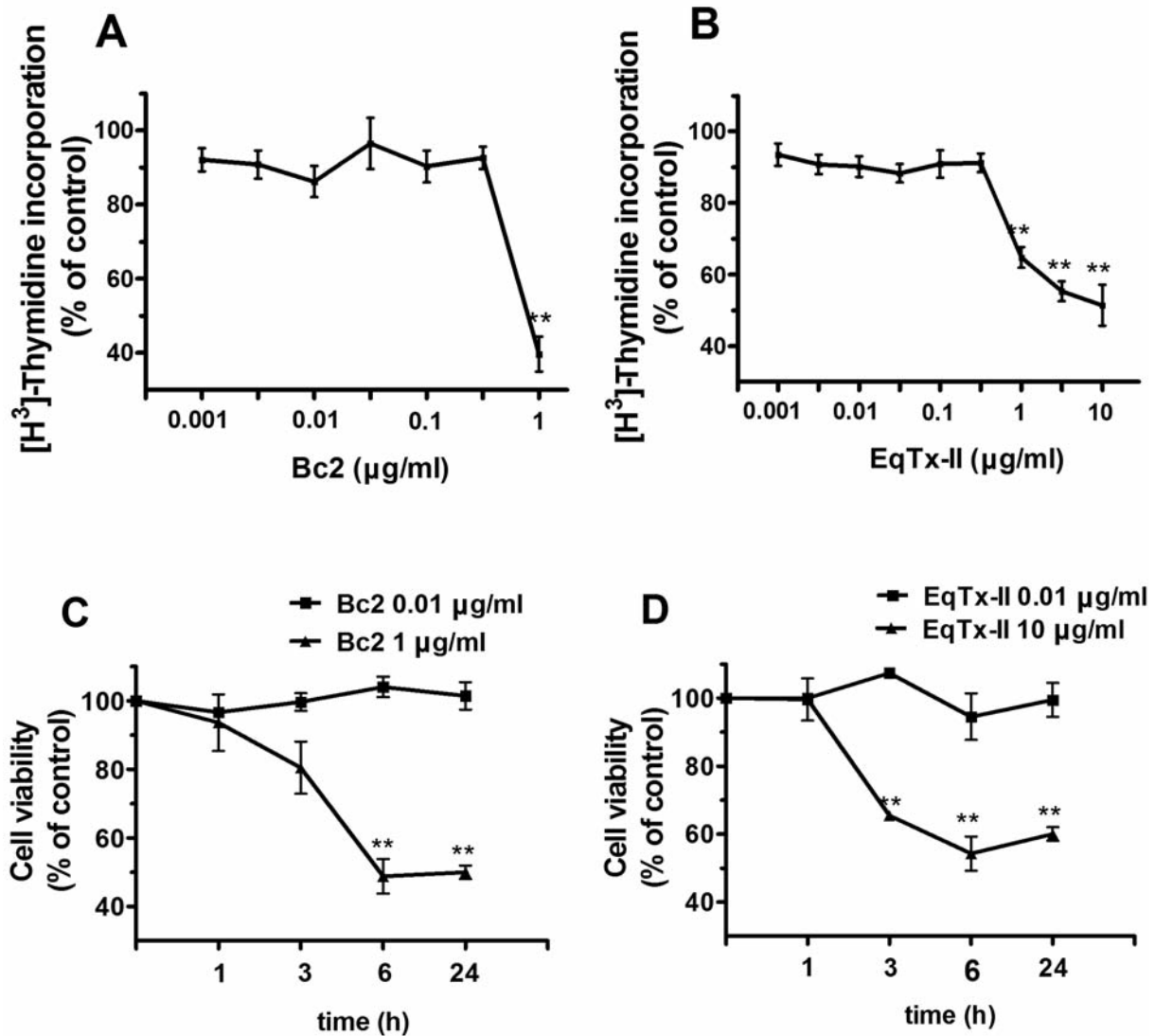


Figure 1. Cytolysis decreases [³H]-thymidine incorporation. Percentage of [³H]-thymidine incorporation in human glioma U87 cells treated with toxin Bc2 (0.001-1 μg/ml, A) or EqTx-II (0.001-10 μg/ml, B) for 24 h. [³H]-Thymidine was given at the last 6 h of incubation. Cellular viability indicated as MTT reduction activity in U87 cells incubated with toxin Bc2 (0.01 and 1 μg/ml, C) or EqTx-II (0.01 and 10 μg/ml, D) for 1 h to 24 h. Data are mean±S.E.M from three independent experiments, each in triplicate. **p<0.01, when compared to untreated cells.

Cytochemical cell staining allowed evaluation of the morphology of U87 cells treated with toxin Bc2 (1 μg/ml) or EqTx-II (10 μg/ml). There was no observation of cell alterations such as cell shrinkage or chromatin condensation that are typical of apoptotic cell death; instead, toxin Bc2 and EqTx-II induced cell lysis (Figure 3A, D and G). To test for the presence of DNA fragmentation *in situ*, one of the hallmarks of apoptosis, TUNEL assay was performed. No difference in the number of TUNEL-positive nuclei was observed among treated and untreated U87 cells (Figure 3 B, C, E, F, H and I). Quantification of TUNEL-positive cells did not exceed 4%

in all experimental conditions. These results suggest that glioma cell viability is compromised by necrosis-like cell death induction.

Involvement of MAPK/ERK, CaMKII and PKC, but not PI3K or PKA pathways on Toxin Bc2 and EqTx-II cytotoxicity. To investigate the cell signaling pathways by which the cytolysins exert their toxicity to U87 cells, cultured cells were pretreated with inhibitors of important key elements of cell death signaling, such as, MAPK/ERK, PKC, PKA, PI3K and CaMKII pathways. Figure 4 shows that the cell death induced by toxin Bc2 (1 μg/ml) or EqTx-II (10 μg/ml), was

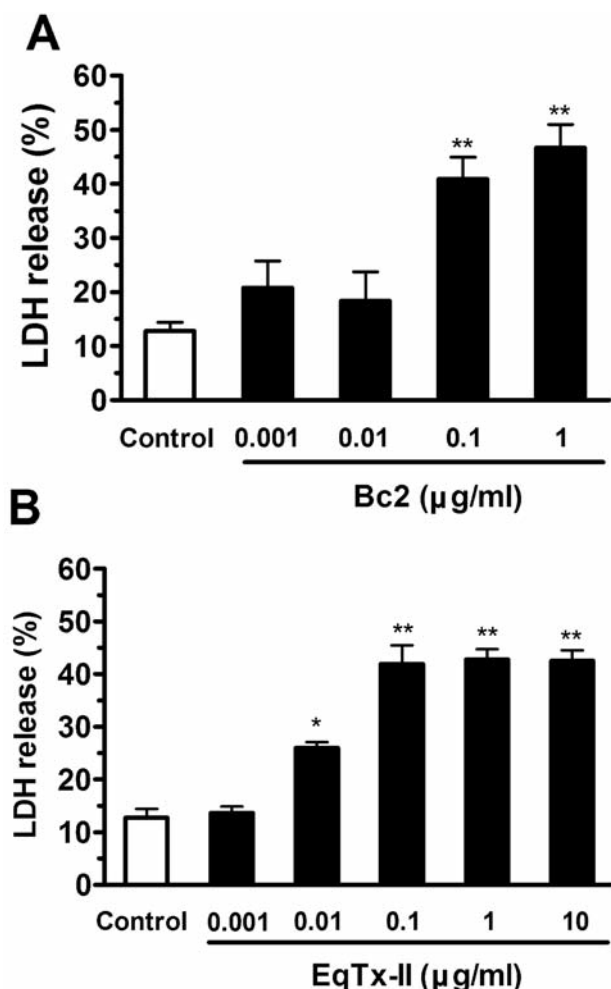


Figure 2. Toxin Bc2 and EqTx-II induce LDH release in U87 cells. Histograms show the percentage of LDH released from U87 cells after incubation with toxin Bc2 (0.001-1 µg/ml) or EqTx-II (0.001-10 µg/ml) for 24 h. Toxins Bc2 (A) and EqTx-II (B) induced a concentration-dependent increase in releasing LDH. Data are mean±S.E.M of three independent experiments in triplicate. * $p < 0.05$ and ** $p < 0.01$, in comparison to the control group in the absence of cytotoxins.

completely abolished by pretreatment with 10 µM PD98059 that blocks the MAPK/ERK pathway. The broad-spectrum PKC inhibitor staurosporine (1 nM) was used to determine the role of PKC in the cytotoxicity induced by cytotoxins. The decrease in cell viability induced by toxin Bc2 and EqTx-II was completely blocked by staurosporine pretreatment (Figure 4). At 1 nM, staurosporine also inhibited the activity of PKA pathway. To confirm whether this pathway is involved in the cytotoxic effect of cytotoxins, H89 (10 µM), a specific PKA inhibitor, was used. The decrease in cell viability induced by Bc2 and EqTx-II was not altered by the pretreatment of U87 with H89 (Figure 4). LY294002 (5 µM), a PI3K inhibitor, did not change the cell death induced by

toxin Bc2 (1 µg/ml) or EqTx-II (10 µg/ml) (Figure 4). The effect of KN-62 (5 µM), a CaMKII inhibitor, on the action of cytotoxins was also tested; the decrease in cell viability induced by both cytotoxins was entirely blocked by KN-62 (Figure 4).

Discussion

New effective anticancer compounds or therapies against gliomas, such as gene therapy, targeted toxins and pore-forming proteins have been investigated (12-15). Recently, new anticancer drugs derived from marine sources have been tested in a large number of experimental and clinical models, with promising results in the treatment of solid- and soft-tissue cancers (24, 25). The present study demonstrated that the cytotoxin toxins Bc2 and equinatoxin-II induced cytotoxicity in U87 human glioblastoma cells with necrosis-like morphology; furthermore, the involvement of MAPK/ERK, CaMKII and PKC pathway signaling was also shown.

Most, if not all cancers, acquire the same set of functional capabilities during their development (26). Among these features, apoptosis resistance is a serious problem to be solved in the treatment of cancer, and more specifically in glioma development. In the setting of a glioblastoma multiforme (the most aggressive/malignant type of glioma, grade IV, WHO), the presence of necrosis is a typical marker of this tumour and, therefore, is used as a diagnostic element (10). In the treatment of cancer patients, drugs capable of inducing apoptosis in tumour cells constitute the major goal. Efforts in the development of necrosis-inducible drugs in gliomas are essential as an alternative mechanism for killing apoptosis-resistant tumours (27, 28). In this context, the present study showed that toxins Bc2 (1 µg/ml) and EqTx-II (10 µg/ml) induce cell death by a non-apoptotic pathway in glioblastoma cells. Moreover, it was previously shown that toxins Bc2 and EqTx-II exert little or no effect on normal astrocytes (8), suggesting that these sea anemone cytotoxins may be used as a tool to induce cell death selectively in cancer cells. Indeed, the combinatory treatment of cytotoxins at low concentration (0.1 µg/ml Bc2 and 0.3 µg/ml EqTx-II) potentiated the cell death induced by several classical chemotherapeutic drugs including Ara-C, doxorubicin and vincristine (8).

Meunier *et al.* (18) demonstrated that EqTx-II (2 µg/ml) causes swelling in neuroblastoma cells due to an increase of cytosolic calcium levels. It was also observed that toxin Bc2 augments the levels of cytosolic calcium (29). Other studies have reported that pore-forming toxins induced apoptosis or necrosis, an effect that is concentration-dependent (30). In general, at high concentrations, cytotoxins form pores that are Ca^{2+} permissive, resulting in necrosis (31). The Ca^{2+} -influx *via* the pore-forming protein toxin Bc2 and EqTxII (29) may be a possible pathway by which cell death is induced. The Ca^{2+} /calmodulin-dependent protein kinase II

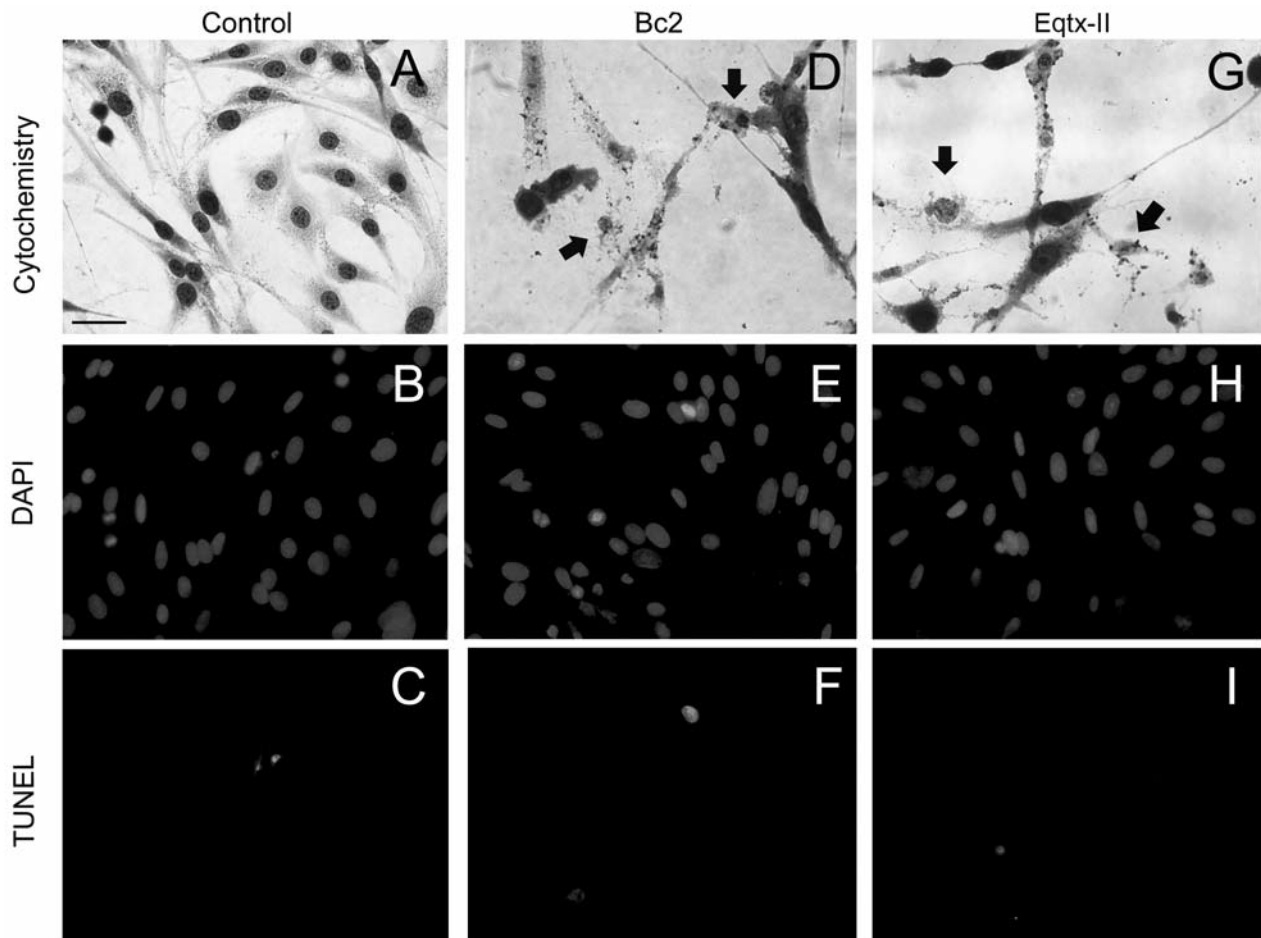


Figure 3. Morphological analysis of U87 human glioma cells in the presence of toxins Bc2 and EqTx-II. U87 cells were incubated with toxins Bc2 (1 $\mu\text{g/ml}$), EqTx-II (10 $\mu\text{g/ml}$), or kept under control conditions for 24 h. Cells were labeled for morphological analysis using Panoptic Kit (A, D, G). TUNEL assay kit was used for apoptosis analysis (C, F, I). Glioma cells from the same field as TUNEL images were counterstained with DAPI for nuclear labeling (B, E, H). Photomicrographics are representative of 3 independent experiments. Arrows in A, D, G point to dead cells. Scale bar in A: 50 μm .

inhibitor, KN-62, blocks the decrease in cell viability induced by Bc2 and EqTx-II. In addition, Ca^{2+} influx can activate PKC, which activates the MAPK/ERK pathway (32). Moreover, the MAPK/ERK pathway can be activated *via* Ca^{2+} or Ca^{2+} /calmodulin in many cell models (33, 34). These results suggest that Ca^{2+} -influx mediated by cytolytins could activate multiple signaling pathways to induce cell death. In accordance with the results of the present study, recent findings suggested that some types of necrotic cell death involve Ca^{2+} -mediated activation of signaling pathways (31, 35).

MAPKs are a family of highly conserved enzymes that orchestrate signal transduction, playing a critical regulatory role in cell proliferation and differentiation (36, 37), as well as promoting cell death in neurons and other cell types (38). In contrast to the substantial experimental evidence that

supports a role for the Ras/Raf/MEK/ERK pathway in oncogenesis, recent studies raised the possibility that activation of this cascade may also suppress tumours (38, 39). Furthermore, several cytolytins, such as pneumolysin, streptolysin and anthrolysin could induce activation of MAPK proteins (40).

In conclusion, the present study showed that toxin Bc2 and equinatoxin-II exert toxicity in human glioma cells, which could be valuable in an apoptosis-resistant cancer context. Moreover, the calcium-induced activation of MAPK/ERK and PKC and CaMKII signaling could be responsible for the cytotoxic effect of sea anemone cytolytins in cancer cells. This study is currently being investigated in additional heterogeneous, low passage glioblastoma cell lines, which may show a varied apoptotic /necrotic response to agents due to the different cell sub-populations.

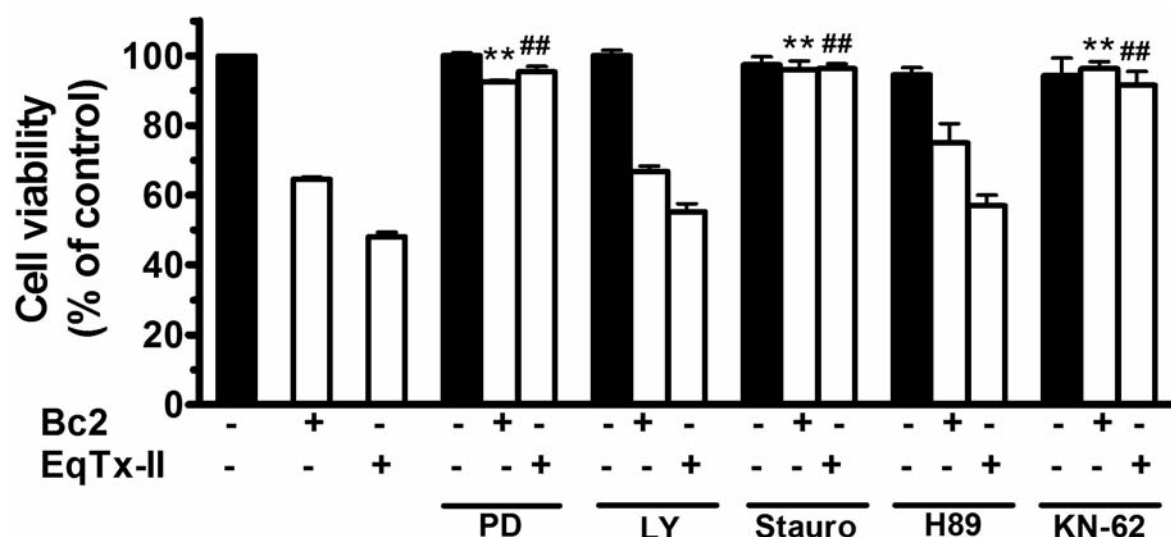


Figure 4. Cell signaling pathways involved in the cytotoxicity of toxin Bc2 and EqtX-II. U87 cells were pretreated with the different kinase inhibitors for 10 min and then treated with toxin Bc2 (1 µg/ml) or EqtX-II (10 µg/ml) for 24 h. Histogram shows the cell viability, as determined by the MTT method, in cultures pretreated with the MAPK/ERK inhibitor PD98059 (10 µM, label PD); the broad-spectrum PKC inhibitor staurosporine (1 nM, label Stauro); the PKA inhibitor H89 (5 µM, label H89); the PI3K inhibitor LY294002 (5 µM, label LY); and the CaMKII inhibitor KN-62 (10 µM, label KN-62). Data are expressed as the mean±S.E.M of three independent experiments in triplicate. **p*<0.01, in comparison to the Bc2 treatment; ##*p*<0.01, in comparison to the EqtX-II treatment.

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