CYTOTOXIC EFFECT OF ICD-85 (VENOM-DERIVED PEPTIDES) ON MDA-MB-231 CELL LINE

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ABSTRACT: Since 1987, when chemopreventive testing programs began, more than 1,000 agents and agent combinations have been selected and evaluated in preclinical studies of chemopreventive activity against various types of cancers. In the present study we aimed to provide quantitative and qualitative characterization of biological and pharmacological activities of ICD-85 on MDA-MB-231 cell line (a highly invasive breast cancer cell line) in order to gain a better understanding of the cytotoxic and apoptotic effects of this compound. For this study, the MDA-MB-231 cell line was used and the effect of ICD-85 was assayed by measuring the activity of the cytosolic enzyme lactate dehydrogenase (LDH), released into the culture medium after membrane damage. Morphological alterations of cells were investigated in the control group and cells incubated with ICD-85 as cytotoxic agent. Results showed, in the test group, that cells incubated with 16 μg/mL of ICD-85 had decreased cytoplasmic branching. Some cells were ruptured and lost the continuity of their surrounding membranes while some had shrunk. Cells incubated with higher doses (above 16 μg/mL) showed similar changes towards cellular normality with more severity. Results obtained from the ICD-85 stability test reveal that the effect of ICD-85 on MDA-MB-231 cell line in culture medium is stable throughout the incubation time period (24 hours). It appears that ICD-85 at higher concentrations acts at the membrane level, which allows the passage of ions down the concentration gradient, resulting in osmotic changes in organelles followed by several unidentified mechanisms leading to cell death. At lower concentrations, it appears that ICD-85 can prevent cell growth by another mechanism, which may be one of the causes for apoptosis in the cell line.

KEY WORDS: venom peptides, MDA-MB-231, anticancer, necrosis, cytotoxic.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION
Although advances have been achieved in adjuvant therapies for breast cancer, many treatments cause significant and potentially life-threatening side effects such as cardiotoxicity (8, 9). A chemoprevention study is defined as the use of specific chemical compounds to prevent, inhibit or reduce cell growth. Since 1987, during chemoprevention testing programs, more than 1,000 agents and agent combinations have been selected and evaluated in preclinical studies of chemopreventive activities, ranging from *in vitro* mechanistic assays and cell-based transformation assays to carcinogen-induced and transgenic animal models (1, 7, 17). New agents are continuously considered for development as chemopreventive drugs, whose selection is based on preliminary efficacy data, mechanistic considerations and potential for improving chemopreventive index (2).

There are reports that present the cytotoxic activity of various snake venoms, *in vitro* and *in vivo*, by employing melanoma and chondrosarcoma cells (5). It was further demonstrated that a purified protein from cobra venom was selectively cytotoxic to cancer cells (4, 6). Silva *et al.* (15) evaluated the action of the venoms from *Crotalus durissus terrificus* and *Bothrops jararaca* on Ehrlich ascites tumors and found that both venoms act directly on tumor cells. They further postulated the various mechanisms involved in the anti-tumor activity. In the present study we aimed to characterize, quantitatively and qualitatively, the biological and pharmaceutical activities of ICD-85 (biologically active peptides from venomous animals) on MDA-MB-231 cell line in order to gain a better understanding of the cytotoxic, apoptotic and necrotic effects of this compound as a chemopreventive agent.

MATERIALS AND METHODS
ICD-85
The active fraction of ICD-85 is a combination of three peptides, ranging from 10,000 to 30,000 Da, derived from the venoms of an Iranian brown snake (*Agkistrodon halys*) and a yellow scorpion (*Hemiscorpius lepturus*). This fraction was formulated and provided by the corresponding author. The ICD-85 peptides were selected based on a study of crude venom cytotoxicity. The crude venom showed antigrowth activity on the MBA-MD231 cell line. Then, the venoms were fractionated, the active peptides were isolated and, subsequentially, tested on the same cell line. Enzymatic characterization of the peptides was not performed.
Cell Line
The MDA-MB-231 breast cancer cell line was kindly provided by Professor Holger Kalthoff from Christian-Albrechts University (Kiel, Germany). These cells were plated in suitable culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, Germany) supplemented with 10% fetal calf serum and 1% L-glutamin plus 0.5% penicillin/streptomycin and incubated at 37°C (10, 11).

Quantification of ICD-85 Cytotoxicity
Cells used in this study were harvested by trypsination (0.5% trypsin in phosphate-buffered saline [PBS]), resuspended in fresh medium and distributed into three (12-well) plates at about 10^4 cells/well (10, 11). After 24 hours of incubation, the cultures were washed with serum free medium (SFM). For dose determination, various concentrations – 1, 2, 4, 8, 16, 32, 64, 128 μg/mL SFM of ICD-85 – were added and the cells were incubated for 48 hours. Control culture was treated with an equal amount of SFM (utilized to avoid the possible interaction between ICD-85 and FBS) (10, 11).

Toxicity was assayed by measuring the activity of the cytosolic enzyme, lactate dehydrogenase (LDH), released into the culture medium after membrane damage. Samples from clarified medium of treated and untreated control wells were taken after intervals of 0.5, 1, 2, 4 and 8 hours of incubation and the LDH activity was measured using the cytotoxicity assay, CytoTox 96® (Promega, USA) associated with a fully automated microplate reader photometer. Damage and morphological changes in the cell line were investigated by light phase contrast microscopy and photos were taken at each evaluation moment.

Stability of ICD-85 in Culture
To examine variations of ICD-85 activity in the liquid medium, culture medium containing ICD-85 at the lowest concentration (1 μg/mL) was incubated at 37°C in a fully humidified atmosphere. After a 24-hour incubation, ICD-85 solution samples were tested on the MDA-MB-231 cell line by measuring LDH.
Statistical Analysis
Data were analyzed using student’s t-test with statistical significance for p < 0.05.

RESULTS
The MDA-MB-231 cells in the control culture samples were homogenously distributed into the culture field. They exhibited polygonal shapes with distinct boundaries and homogenous or slightly granulated cellular contents. The cells were thin and elongated with tapered ends in cytoplasmic branches (metastatic branches). In test groups, cells incubated with smaller doses (less than 8 μg/mL of ICD-85) were almost similar in morphological appearance to the control cells; and most MDA-MB-231 cells conserved their common characteristic structure, except for the occurrence of cellular swelling and diminished cytoplasmic branches (Figure 1 – A and A1). Cellular swelling and cytoplasmic branch reduction occurred after incubation with the 16 μg/mL dose. Additionally, some cells were ruptured and cell number decreased (Figure 1 – B and B1). Cells incubated with higher doses (above 16 μg/mL), presented similar changes towards normality. In this case, several cells appeared rounded rather than elongated. Furthermore, few cells still maintained their MDA-MB-231 common appearance.
Results obtained from the ICD-85 stability test revealed that ICD-85 is stable throughout the incubation time period (24 hours) in the culture medium and that its cytotoxic effects on the MDA-MB-231 cell line are significant. LDH released from cultured cells and exposed to a biomaterial provides a sensitive and accurate marker for cellular toxicity. As shown in Table 1, ICD-85 induced identical dose-response effect on MDA-MB-231 cells, releasing 9, 13, 15, 19, 23, 29, 30 and 34 IU of LDH after 30 minutes (at respective concentrations of 1, 2, 4, 8, 16, 32, 64 and 128 μg/mL).

Table 1. Comparison of LDH activity in cultured media 30 min. after incubation of MDA-MB-231 cell line treated with ICD-85

<table>
<thead>
<tr>
<th>Groups</th>
<th>ICD-85 concentration µg/mL</th>
<th>ICD-85 treated cells LDH activity (IU)</th>
<th>p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>9</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>9 ± 3</td>
<td>NS</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>13 ± 5</td>
<td>NS</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>15 ± 5</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>19 ± 4</td>
<td>p &gt; 0.01</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>23 ± 5</td>
<td>p &gt; 0.001</td>
</tr>
<tr>
<td>7</td>
<td>32</td>
<td>29 ± 4</td>
<td>p &gt; 0.001</td>
</tr>
<tr>
<td>8</td>
<td>64</td>
<td>30 ± 5</td>
<td>p &gt; 0.001</td>
</tr>
<tr>
<td>9</td>
<td>128</td>
<td>34 ± 7</td>
<td>p &gt; 0.001</td>
</tr>
</tbody>
</table>

Note: all groups were compared with Group 1 (untreated cells). The tests were repeated six times. NS: not significant.

DISCUSSION
Evidence from basic research and from experiments on animal models and cell culture systems supports the concept that several compounds, including natural products, may offer protection against organ site carcinogenesis (3, 16). These biological products or natural phytochemicals, either as single agents or as adjuvants, may represent valuable lead compounds for preventive or therapeutic interventions (6).

Crude snake venoms and their isolated peptides have proven effective cancer cell inhibitors (12-14).

The MDA-MB-231 cell line was employed in the current study due to its extremely invasive behavior (10, 11). This investigation showed that the damage induced in the cell line by ICD-85 is direct. The cytotoxicity was remarkable and cell survival was reduced by the two highest concentrations (64 and 128 µg/mL). Considering the rapid effects observed within 30 minutes – including the lysis of organelle membranes that immediately caused cell death – we suggest that this biological product (ICD-85) acts at the membrane level at high concentrations. The cytotoxicity assay that measured
LDH activity can also detect whether cells and apoptotic bodies remain enclosed by intact plasma membrane, and thus, can be used to differentiate between apoptosis and necrosis. Since ICD-85, at high concentrations, acts at the membrane level, it allows the passage of ions down their concentration gradient, resulting in osmotic changes in organelles followed by several unidentified mechanisms leading to cell death. This process may be involved in cell line apoptosis, as a result of ICD-85 treatment. In this respect, apoptosis and other forms of cell death could be set in motion by diverse stimuli such as chemical toxicity, radiation or genotoxic damage.

The ICD-85 effect was also tested on two additional cell lines, human leukemia (HL-60) and Vera cell line. The results of this peptide on both were almost similar to MDA-MB-231 (unpublished data). At 7 \( \mu \text{g/mL} \) concentration, on the HL-60 cell line, ICD-85 could transform the growth trends towards normality. The ICD-85 effect on normal cells (MRC5), at low concentrations (5, 10 and 15 \( \mu \text{g/mL} \)), after 24 hour incubation, showed no significant cell damage. However, when concentration increased and was above 20 \( \mu \text{g/mL} \), the cytotoxic effect of ICD-85 was evident. It appears that ICD-85 acts selectively on cancer cells at low concentration.

Despite the great advances in understanding the morphological and biochemical alterations associated with substances that can induce cell injury, the knowledge acquired is still insufficient to establish which changes lead to cell death and which are secondary disturbances.

The current study aimed to clarify some of the ICD-85 cytotoxic effects on cultured cell lines. Several conclusions can be reached from this study. It is clear that the cytotoxic effects are dose- and time-dependent, and ICD-85 kills cells by different mechanisms. The membrane damage is more prominent and clear in the early stages of toxicity, while other forms of cell damage – including swelling, rupture or necrosis – occurred in the later stages. Recently, local injection of ICD-85 at breast cancer targets was carried out and produced anti-angiogenesis features, lifespan increase and tumor size decrease in mice presenting induced breast cancer (under publication). Based on the results obtained herein and in in vivo studies, we hope that a new biologically active drug against cancer will be developed.

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