Selective Cytotoxicity of α-Santonin from the Persian Gulf Sponge Dysidea Avara on Pediatric ALL B-lymphocytes via Mitochondrial Targeting

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Abstract

Background: Acute lymphoblastic leukemia (ALL) is one of the most dominant malignancies among children, characterized by production of immature and dysfunctional blasts which are resistant to cytotoxic chemotherapeutic agents. Therefore, research protocols are currently focusing on discovery of novel anti-cancer agents to enhance survival rates and decrease unwanted side effects. Approximately two-thirds of the planet is covered by oceans with a massive range of marine organisms of interest to scientists in pharmaceutical fields. Methods: Among marine resources, sponges are known to have beneficial effects in the treatment of numerous malignancies. One fraction of crude extracts containing α-Santonin was made from the Persian Gulf marine sponge, Dysidea avara, and investigated for anticancer effects. Results: Treatment of ALL B-lymphocytes with the Dysidea avara extract caused augmentation in ROS generation, decline in mitochondrial membrane potential, mitochondrial swelling, release of cytochrome c from mitochondria and activation of caspase-3 only in mitochondria isolated from B-ALL lymphocytes. Conclusion: In brief, our results suggest that Dysidea avara extracts may selectively induce apoptosis in malignant pediatric lymphocytes.

Keywords: B-ALL lymphocyte- Dysidea avara- mitochondria- apoptosis, Persian Gulf

Introduction

Cancer is one of the main causes of death around the world and according to WHO estimation in 2020 almost 10.3 million people would die because of this incurable malignancy.

Pediatric Acute lymphoblastic leukemia (ALL) is one of the life-threatening malignancies which affects children with a high incidence below the age 6 years old (Schwab and Harrison, 2011; Roganovic, 2013). The complete eradication of the injured and dysfunctional lymphoid cells without alteration in normal precursors is desirable and the main objective of each therapeutic protocol. Chemotherapy is the first line treatment strategy, however, the standardized chemotherapy regimen have a serious or permanent side effects. Due to the undesired toxic effects of chemical compounds, many new naturally occurring compounds are introduced as a replacement (Pui et al., 2004; Schwab and Harrison, 2011).

Marine organisms are an abundant source of bioactive molecules, and the Persian Gulf is the rich environment which contains a huge number of various species. Among marine resources, more than 15,000 species of marine sponges are known worldwide (Thomas et al., 2010), and they considered as a beneficial natural source in pharmaceutical studies for many years (Simmons et al., 2005). A lot of studies on marine sponges indicated that sponge Dysidea avara, have cytotoxic effects on cancer cell lines; thus it is suggested that marine drugs can potentially be used as a beneficial medicine in cancer therapy. These studies have just concern about clinical consequences. However, in this study, Fractions containing α-Santonin was investigated for cytotoxic activity in human B-ALL lymphocytes comparing with healthy normal cells. Here in, we emphasis on the mechanism of Dysidea avara fractions containing α-Santonin to onset the intrinsic pathway of apoptosis.

By consideration of mitochondrial parameters such as reactive oxygen species (ROS) generation, mitochondrial membrane potential (MMP) decline, mitochondrial swelling, release of cytochrome c, and caspase-3 activity, we demonstrated that Dysidea avara fractions containing α-Santonin selectively activate apoptosis through mitochondrial pathways in human leukemic cells.
Materials and Methods

Chemicals

Trypan blue, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), Rhodamine123, bovine serum albumin (BSA), N-(2-hydroxyethyl) piperazine-N′- (2-ethanesulfonic acid) (HEPES) were purchased from Sigma-Aldrich Co., RPMI1640 and FBS (Fetal Bovine serum) were purchased from Gibco, Life Technologies, Grand Island, NY. Ficoll-paque PLUS was obtained from Ge Healthcare Bio-Science Company. Marine sponge extract and fractions containing α-Santonin were prepared in Persian Gulf and Oman Sea ecological research organization, Bandar Abbas, Iran.

Sampling and identification

Dysidea avara was collected by scuba diving in June 2016; from reef those habitats at depths of 20-25 m in Hengam Island on the Persian Gulf. After identification of species the samples were lyophilized and dried samples used for extraction.

Extraction

The sponge dried sample powder extracted with acetone solvent. After 72 hours of soaking in acetone the solvent filtered and acetone evaporated to dryness, at low pressure at 35- 40°C by using Rota vapor (Çitoğlu and Acıkara, 2012).

Isolation of Mitochondria

Mitochondria were isolated from the ALL B-lymphocytes by mechanical lysis and multiple centrifugations. Briefly, B-lymphocytes were washed with cold PBS and centrifuged at 450× g. The pellet was re-suspended in cold isolation buffer. Non-lysed cells and nuclei were spun down by centrifugation at 1,000 × g for 10 minutes. The supernatant was further centrifuged at 20,000 × g for 25 minutes. The pellet, designated as the mitochondrial fraction, was suspended in assay buffer. The accuracy of mitochondrial isolation was determined by succinate dehydrogenase measurement (Rotem et al., 2005).

Succinate Dehydrogenases Activity Assay

The activity of succinate dehydrogenases was examined by estimation of MTT reduction (Berridge and Tan, 1993). The colorimetric assay was performed by ELISA reader (Tecan, Rainbow hermo, Austria).

In this case we used an internationally accepted protocol using at least three different concentrations as IC50/2 (sub-toxic concentration), IC50 (toxicity threshold concentration) and 2×IC50 (very toxic concentration). In our research the IC50 determined for sponge fractions containing α-Santonin on isolated B-ALL lymphocyte mitochondria was approximately 300µg/ml and therefore we used 150µg/ml as sub-toxic and 600µg/ml as very toxic concentrations. To determine the IC50 of sponge fractions containing α-Santonin which defined as a concentration which decreased the activity of mitochondrial succinate dehydrogenase down to 50% and considered as toxicity threshold in accelerated cytotoxicity mechanisms screening (ACMS) technique, we applied 7 different concentrations obtained from literature review (shown in Figure 3) on isolated B-ALL lymphocyte mitochondria using MTT assay. In order to determine IC50 value for our investigated compound, dose–response curves were plotted and the IC50 determination was based on a regression plot of 7 different concentrations (data and curves not shown).

Determination of ROS formation

The mitochondrial ROS formation was performed using the fluorescent dye DCFH-DA. Briefly, isolated mitochondria were placed in respiration buffer, and then added different concentrations of sponge fractions containing α-Santonin at 37 °C for 60 minutes. Finally, the fluorescence intensity was measured by fluorescence spectrophotometer at an excitation and emission wavelength 488 nm and 527 nm respectively (Rezaei et al., 2014).

Determination of Mitochondrial Membrane Potential (MMP)

MMP assayed by a fluorescent dye, rhodamine 123. The mitochondrial fractions were incubated with 10 µM of Rhodamine 123 in MMP assay buffer and then various concentrations of sponge fractions containing α-Santonin at 37 °C were added to this suspension.
The fluorescence intensity was monitored using fluorescence spectrophotometer at the excitation and emission wavelength of 490 nm and 535 nm, respectively (Hosseini et al., 2013).

**Determination of Mitochondrial Swelling**

Mitochondria suspensions were incubated in in swelling buffer supplemented with 1 mg/mL rotenone and 10 mmol/L succinate at 37 °C.

After 10 minutes of pre-incubation, various concentrations of sponge fractions containing α-Santonin were added. Mitochondrial swelling was measured spectrophotometrically (at 540 nm) within 60 minutes (Rotem et al., 2005).

**Identification of α-Santonin Compounds**
The α-Santonin with the chemical formula C_20H_30O_3 (Figure 2), belonging to the teriterpenoid group identified with 92% purity in fraction 50-55.

**Succinate Dehydrogenase Activity Assay**

In this investigation, MTT assay was used to evaluate the impact of sponge fractions containing α-Santonin on mitochondrial succinate dehydrogenase. As shown in Figure 3 this extract can effectively inhibit succinate dehydrogenase activity in isolated ALL mitochondria without any significant effect on normal mitochondria.

**ROS formation assay**

As a consequence of the fact that Radical Oxygen Species (ROS) formation is a critical point in apoptosis initiation, we tested if sponge fractions containing α-Santonin could change these species level in ALL and healthy mitochondria. As shown in Figure 3 this extract can effectively inhibit succinate dehydrogenase activity in isolated ALL mitochondria without any significant effect on normal mitochondria.

**Mitochondrial Membrane Potential (MMP) assay**

We evaluated the impact of sponge fractions containing α-Santonin on mitochondrial membrane potential on both healthy and ALL samples. Treatment with different concentrations (150, 300, and 600µg /ml for...
60 minutes) significantly (p< 0.0001) reduced MMP in B-ALL mitochondria. Conversely, the IC$_{50}$ concentration (300 µg/ml) did not cause any significant effects on mitochondrial potential in healthy samples (Figure 5).

Mitochondrial swelling assay

Mitochondrial swelling followed up by detecting the absorbance of mitochondria within 60 minutes at 540 nm. As demonstrated in Figure 6, following addition of different concentrations (150, 300, and 600 µg/ml) of
Toxicity of α-Santonin on ALL Lymphocyte

Sponge fractions containing α-Santonin, a great swelling in mitochondria obtained from B-ALL lymphocytes was observed. However, sponge fractions containing α-Santonin at IC_{50} concentration did not cause mitochondrial swelling in healthy samples.

**Determination of Cytochrome c Release**

As shown in Figure 7 sponge fractions containing α-Santonin at IC_{50} (300µg/ml) significantly (p < 0.01) caused release of cytochrome c into incubation buffer. Our results revealed that sponge fractions containing α-Santonin can cause mitochondrial permeability transition (MPT) pore opening and cause cytochrome c expulsion from mitochondria.

**Determination of Caspase-3 Activity**

As shown in Figure 8, sponge fractions containing α-Santonin at IC_{50} (300 mg/ml) significantly (p < 0.0001) enhanced the activity of caspase-3, the critical apoptosis mediator, in B-ALL but not in healthy lymphocytes.

**Discussion**

Cancer is classified in the category of complicated diseases with involvement of multiple factors such as genetic, lifestyle, and age (Schnekenburger et al., 2014). Traditional chemotherapeutic agents applied their anticancer effects by the direct cytotoxic mechanism. Nevertheless, these cytotoxic effects are not always restricted to malignant cells and are usually accompanied by several side-effects. Therefore, the search for more selective anticancer compounds is the main goal of medicine (Rady, 2014). Literature documents reveal that somenaturally occurring molecules which isolated from marine sponges have exhibited antineoplastic properties (Müller et al., 1985; Sawadogo et al., 2015). α-Santonin, is one of these natural compounds. Recent studies have been demonstrated antitumor properties of marine sponge extract on many types of cancers (Müller et al., 1985; Schwartsmann et al., 2001).

In this study, we investigated that marine sponge
extract can trigger apoptosis through mitochondrial pathway.

Our result showed that incubation of mitochondrial suspension with sponge fractions containing α-Santonin in IC<sub>50</sub> concentration significantly increased ROS formation. These reactive species may promote MPT (mitochondrial permeability transition) pore by oxidation of thiol groups which induced ΔΨ<sub>m</sub> collapse, mitochondrial swelling and finally release of the mitochondrial apoptotic factor. Our findings also revealed that sponge fractions containing α-Santonin can induce mitochondrial swelling in a time and concentration-dependent manner only in ALL B-lymphocyte. We, therefore, concluded that sponge fractions containing α-Santonin has a considerable role in initiating apoptosis on mitochondria isolated from ALL B-lymphocytes without significant effect on normal healthy mitochondria.

In conclusion, the anticancer activity of sponge fractions containing α-Santonin could associate with its apoptosis induction of ALL cells through mitochondrial pathway. Based on our findings, sponge fractions containing α-Santonin may be an efficacious candidate as a selective naturally occurring chemotherapeutic agent against pediatric lymphoblastic ALL.

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