Abstract. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising anticancer agent, as it can kill tumor cells selectively. In our search of bioactive natural products to overcome TRAIL-resistance, we isolated 47 actinomycete strains from different sediments and seawater samples collected from the Red Sea coast in Egypt and found four crude extracts (EGY1, EGY3, EGY24 and EGY34) displaying TRAIL sensitizing activity in the resistant breast cancer cell line MDA-MB-231. None of these crude extracts exhibited cytotoxic effect on normal mouse embryonic fibroblasts (MEF), with the exception of EGY34. Analysis of the signaling pathways underlying the sensitization of MDA-MB-231 cells to TRAIL-induced apoptosis, by western blotting, revealed that all crude extracts facilitated initiator caspase-8/-10 activation upon TRAIL stimulation, but that in addition, EGY3 and EGY34, alone, induced strong ER-stress activation, with the appearance of BiP in the cytosolic extracts. Our results pave the way to the discovery and the development of marine-derived drugs for cancer therapy.

Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a type II transmembrane protein belonging to the tumor necrosis factor (TNF) superfamily. It is expressed on the surface of certain immune cells including T-lymphocytes and natural killer (NK) cells. TRAIL plays a central role in immune surveillance. Owing to the fact that it induces selective apoptosis in tumor cells, but not normal cells, TRAIL has been considered as a promising therapeutic anticancer agent (1,2). The apoptotic signaling pathway regulated by TRAIL is initiated by its binding to agonist death receptors, namely DR4/TRAIL-R1 and DR5/TRAIL-R2. Oligomerization of these receptors allow the recruitment of the adaptor protein Fas-associated death domain (FADD), which subsequently stimulates the formation of death-inducing signaling complex (DISC) through recruitment of the pro-caspase-8 (3,4). Activation of pro-caspase-8 within the DISC, by proximity, activates either a cascade of caspase-cleavage leading to activation of caspase-3, -6, and -7 (extrinsic pathway) or stimulates the mitochondrial pathway (intrinsic pathway) via cleavage of the BH3-interacting domain death agonist (Bid) to truncated Bid (tBid)/caspase-3, inducing an irreversible cell death (5). Despite potent anticancer activity, resistance to TRAIL-induced apoptosis has been reported in several malignant cells (6-12). The mechanism by which the tumor cell gains resistance to TRAIL mainly include i) a deficiency of DR expression, ii) overexpression of anti-apoptotic proteins, or iii) competition of the decoy receptors (DcR1/TRAIL-R3 and DcR2/TRAIL-R4) for TRAIL binding (13-15). However, conventional or non-conventional chemotherapy can restore TRAIL sensitivity (16-18).

Marine environment encompasses a great variety of microorganisms living in extreme conditions such as high salinity, pressure and temperature. These organisms were reported to produce unique and structurally novel secondary metabolites allowing their survival in such conditions (19). Marine actinomycetes are considered as an unexplored source of biologically active secondary metabolites including antibacterial, anti-fungal, anti-malarial, anti-inflammatory, and antitumor (20-25). Several natural products derived from terrestrial and marine actinomycetes were investigated for their TRAIL-resistance overcoming activities and the molecular mechanisms that trigger the induction of apoptosis were determined (25-30). In this study, we investigated TRAIL-resistance overcoming activity of several crude extracts of marine actinomycetes isolated from the Red Sea. In TRAIL-resistant MDA-MB-231 breast cancer cells, four crude extracts obtained from Streptomyces sp. (EGY1, EGY3, EGY24, and EGY34) showed selective TRAIL synergistic activity, but had no effect on the normal mouse embryonic fibroblast (MEF). Analyzing the signaling pathways triggered by the
co-treatment indicate that these crude extracts possibly act through two distinct mechanisms; either through i) activation of caspase-8 and -10 pathways or ii) induction of ER-stress via stimulation of ER-stress sensors, BiP. Our results are expected to provide new insight into the development of lead marine-derived structures against cancer.

Materials and methods

Collection of samples. Thirty samples of sediments, seawater and sands were collected from different parts of Sharm El-Sheikh, South Sinai, Egypt. The collected samples were kept in 50 ml sterile Falcon tubes and preserved in refrigerator for further study.

Isolation of actinomycetes. Wet sediment/sand (1 g) was dispersed in 9 ml of sterilized water. The samples were vortexed for 2 min and subjected to heat treatment in a water bath at 60°C for 10 min to eliminate non-spore-forming bacteria. Following serial dilution (10⁻¹, 10⁻² and 10⁻³) of the suspension with sterile water, a 100 μl aliquot was spread on humic acid-vitamin agar (31): humic acid (1.0 g/l), Na₂HPO₄ (0.5 g/l), used to inoculate 2x100 cm³ cultures, chunks of well grown agar plate of each strain were added to each well. After 24 h incubation, the culture media was removed and the cells were washed with 100 μl PBS. Cells that remained attached were fixed by adding 100 μl of 70% ethanol. The plate was then incubated at room temperature for 1 h to remove ethanol and 100 μl of methylene blue dye was added. The plates were incubated at room temperature for 15 min. To remove the excess of dye, the plate was washed three times with tap water and then dried for 2 h at 37°C. Dye was eluted from the attached cells by adding 100 μl of 0.1 M HCl in each well and then incubated for 5 min at room temperature. The developed blue color was measured using an ELISA reader at 630 nm.

To investigate whether the crude extracts have a synergistic effect with TRAIL, MDA-MB-231 breast cancer cells were treated with the crude extracts at various concentrations (0.04, 0.1, 0.2, 0.3, 0.6 and 1.2 mg/ml) for 24 h, followed by stimulation with TRAIL at final concentration of 250 ng/ml for an additional 24 h. Controls referred to wells containing only cells and medium with and without 10% DMSO.

Detection of apoptosis. The MDA-MB-231 cells (1x10⁵ cells per well) were seeded in a 12-well plate, treated with different doses of crude extracts (EGY1, EGY3, EGY24, EGY34) and TRAIL (250 ng/ml) then incubated for 16 h at 37°C. The cells were harvested by centrifugation and stained with Annexin V and propidium iodide (PI) according to the manufacturer’s instructions (AbCys SA). Quantification of apoptosis-induced by the crude extracts with and without TRAIL was analyzed by flow cytometry using a FACScan II flow cytometer (BD Becton-Dickinson).

Western blot analysis. Approximately 1x10⁶ of the cells were seeded in 6-well plates, treated with the crude extracts, and then incubated for 24 h. The cells were stimulated with TRAIL (250 ng/ml) and incubated for 24 h. Afterwards, the cells were collected and lysed in lysis buffer (1% NP-40, Tris-HCl, 3 M NaCl, 5% glycerol). The concentration of protein was determined by Bradford reagent (Bio-Rad, Marnes-la-Coquette, France). Proteins were resolved by electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gel and transferred by electro-blotting to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The membranes were incubated overnight with the following primary antibodies. Anti-TRAIL-R1 (cat# AB16955) and TRAIL-R2 (cat# AB16942) antibodies were purchased from Chemicon (Millipore, Molsheim, France), antibodies against caspase-3 (clone 8G10), caspase-8 (clone 5F7) and caspase-10 (clone 4C1) were all from Medical & Biological Laboratories (Clinisciences, Montrouge, France). Anti-BIP (cat# 3177) and CHOP (cat# 2895) were obtained from Cell Signaling Technology (Ozyme, St. Quentin Yvelines, France).
France). Anti-FLIP antibody (clone Dave-2) was from Adipogen (Coger, Paris, France), anti-cleaved lamin A/C Asp230 (cat# 3596-1) from Epitomics (Abcam, Paris, France). Anti-GAPDh (clone 0411) antibody was from Santa Cruz biotechnology (Clinisciences, Nantere, France). Anti-actin antibody (cat# 4970) was from Cell Signaling Technology. hRP-conjugated anti-rabbit or mouse secondary antibodies were from Jackson Immuno Research (Interchim, Montluçon, France), hRP-conjugated anti-mouse IgG1-, Ig2a- and Ig2b-specific antibodies were from Southern Biotech (Clinisciences). After 3 washes in PBS-Tween 0.5% for 10 min, membranes were incubated for 1 h with the corresponding secondary antibody and washed as above. Blots were developed using the Advansta westernBright chemiluminescence substrate according to the manufacturer’s protocol (Diagnostics, Blagnac, France) followed by image processing using the Bio-Rad imaging system.

### Results

**Cytotoxic effect of crude extracts and their sensitization to TRAIL in breast cancer cells.** Forty-seven actinomycete strains were isolated from different areas of the Red Sea shore of Sharm El-Sheikh, South Sinai, Egypt. Microscopic observations revealed that both aerial and vegetative hyphae were abundant and well developed. The color of aerial and substrate mycelium of 21 strains varied from yellow to red. As we are interested in pigmented secondary metabolites, the cytotoxic effect of 21 crude extracts at concentration of 0.1 mg/ml was screened sequentially with and without TRAIL (250 ng/ml) in MDA-MB-231 breast cancer cells (Table I). The crude extracts that produced approximately >20% inhibition along with TRAIL than the agent alone were selected for further investigation. Four extracts corresponding to actinomycetes EGY1, EGY3, EGY24 and EGY34 showed synergistic effects with TRAIL. Partial 16S rRNA sequence analysis revealed that the effective strains are new member of the genus *Streptomyces* sp., indicating <98.2% sequence similarities (Fig. 1). The 16S rRNA gene sequence of EGY1, EGY3, EGY24, and EGY34 was submitted to GenBank with the accession numbers, KX218228, KP895876, KP792816, and KP792818, respectively.

<table>
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<th>Cell viability (%)</th>
<th>Diff. (%)</th>
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**Table I. Cytotoxic effects of 21 marine actinomycete crude extracts with and without TRAIL (250 ng/ml) on TRAIL-resistant MDA-MB-231 breast cancer cell line.**

The sensitizing effect of the crude extracts of EGY1, EGY3, EGY24 and EGY34 to TRAIL-induced apoptosis in MDA-MB-231 breast cancer cell line in both sequential and combined treatment with TRAIL was tested. Cells were incubated with various concentrations of the crude extracts (0.04, 0.1, 0.2, 0.3, 0.6 and 1.2 mg/ml) for 24 h. A significant concentration-dependent decrease of the cell viability of MDA-MB-231 cancer cell line was observed (Fig. 2A). When TRAIL (250 ng/ml) was sequentially added for an additional 24 h, cell viability was significantly reduced in cells prestimulated with these crude extracts as compared to TRAIL alone. Extracts EGY1, EGY3, EGY24 and EGY34 induced a marked decrease in cell viability in the presence of TRAIL (250 ng/ml), compared to TRAIL alone (Fig. 2A). Combined treatments were also assessed and cells were treated with various concentrations of the crude extracts and TRAIL (250 ng/ml) for 16 h (Fig. 2B). Of note, TRAIL sensitizing activity of these crude extracts, even at lower concentrations,
was greatly enhanced. This gives a slight indication that the synergistic effect-induced by both sequential and combined treatment may be due to early apoptotic events including DISC assembly and subsequent activation of initiator caspases.

Phenotypic changes of cells pretreated with the crude extracts and after sequential stimulation with TRAIL were analyzed by microscopic investigation (Fig. 3). Pretreatment of MDA-MB-231 cells with the identified crude extracts induced
an early apoptosis, observed by the appearance of granulated cellular lumen, indicative of apoptotic bodies. On the other hand, cells stimulated with both crude extracts and TRAIL showed marked apoptosis in which the condensation of the genetic material in the cell center was clearly detected. In terms of selectivity, the cytotoxic effect of the crude extracts on normal mouse embryonic fibroblast (MEF) cell line was tested (Fig. 4). The crude extracts showed no cytotoxicity toward the MEF cell line. Only crude extract EGY34 induced a significant cytotoxicity at the higher concentrations (2.5 and 5.0 mg/ml) compared to the non-treated cells.

Flow cytometric analysis of apoptosis. Annexin V and PI staining were used to measure apoptosis induced by the crude extracts alone and in combination with TRAIL (Fig. 5). Cells were treated with the crude extracts at the lowest effective concentrations and TRAIL (250 ng/ml) for 16 h. The results correlated with the increase in the percentage of apoptosis compared to the non-stimulated cells. Flow cytometric analysis detected a slight increase in the percentage of the apoptotic cells in the control with and without TRAIL from 6.2 to 18.7% confirming the resistance of the MDA-MB-231 cells to TRAIL-induced apoptosis. The highest apoptotic effect was measured for the combined treatment of crude extracts EGY3 and EGY24 with TRAIL. These combinations increased apoptosis in MDA-MB-231 cells by approximately 5- and 2-fold, respectively. Some extracts, including EGY1, EGY24 and EGY34 induced, alone, a large amount of cell death as
Evidenced by Annexin V and PI staining (Fig. 5). Stimulation in the presence of TRAIL, however, and consistent with cell viability assays (Fig. 2), further increased cell death in resistant MDA-MB-231 cells, as evidenced with EGY3 and EGY24 extracts (Fig. 5).

Analysis of the signaling pathways triggered by the combined treatment. The molecular mechanisms by which the identified crude extracts trigger the sensitization of the breast cancer cells to TRAIL-induced cell death were explored by western blot analysis (Fig. 6). All crude extracts facilitated activation of the initiator caspase-8/10 as a result of combined treatment with TRAIL. Consistent with the increase in Annexin V staining (Fig. 5), an increase in cleaved lamin A/C was detected in cells stimulated with TRAIL and EGY1 or EGY34, but less so with EGY3 and EGY24. Noteworthy, some crude extracts were also able to induce an ER stress response. Likewise, as compared to serum starvation, EGY3 and EGY34, but not EGY1 and EGY24 induced by the appearance of the ER-stress sensor, binding immunoglobulin protein (BiP). These results therefore suggest that the actinomycete crude extracts identified here exhibit TRAIL-sensitizing activity, and are likely to act at least at the level of the TRAIL DISC.

Discussion

The hallmark features for initiation and progression of cancer is mainly assigned to the defect in the apoptotic signaling pathways. Despite the potent anticancer effect of TRAIL, several tumor cell lines including lung and breast cancer cells were found to possess resistance to TRAIL-induced apoptosis (35). Overcoming TRAIL-resistance by combined treatment using a chemotherapeutic agent is considered as a promising drug target in the treatment of cancer. Marine microorganisms, particularly actinomycetes still remain a rich source for the discovery of marine natural products. They are a group of bacteria, reported to produce great variety of bioactive secondary metabolites and represent a focal point in the search for novel antimicrobials and anticancer agents (36,37). The cytotoxic effect of several plant and marine sponge extracts against various tumor cell lines was reported (38-43). However, the anticancer effect of crude extracts isolated from marine microorganisms has not been studied yet.

In this study, 21 crude extracts of marine actinomycetes isolated from the Red Sea were found to produce yellow to red pigments based on the morphological properties. Actinomycetes are characterized by the production of various types of colored compounds (i.e. anthraquinones and phenazines) with TRAIL-resistance overcoming activity in different cancer cell lines (25,26,28,29). Therefore, the TRAIL-resistance overcoming activities of the 21 crude extracts were investigated. Four crude extracts (EGY1, EGY3, EGY24, and EGY34) revealed a significant sensitizing effect to TRAIL-induced cell death. The extracts exhibited significant cytotoxic and synergistic effect by combined treatment rather than by sequential treatment.
with TRAIL. The significant impact of the crude extracts on the sensitization of the breast cancer cells to TRAIL-induced cell death was also confirmed by flow cytometry. It was shown that the combined treatment of the crude extracts potentially enhanced the effect of TRAIL by increasing the reduction of the cell viability. We are still in the characterization phase of the identified crude extracts represented in the purification and structural elucidation of the bioactive components as potent therapeutic agents against breast cancer.

Analyzing the molecular mechanisms for the restoration of apoptotic cell death by western blotting initially revealed two possible signaling pathways; i) stimulation of extrinsic pathway and DISC formation via activation of the initiator caspase-8/-10 and ii) stimulation of ER-stress-induced cell death. The combined treatment of MDA-MB-231 cells with the identified crude extracts and TRAIL was assumed to sensitize the cells to TRAIL-induced apoptosis by the activation of the ER-stress sensor BiP as a result of the combinatorial treatment of MDA-MB-231 cells with EGY3, EGY34, and TRAIL. Our results are expected to provide new insight into the development of lead compounds against breast cancer and other types of cancer.

Acknowledgements

This work was supported by the Science and Technology Development Fund (STDF), Egypt (grant no. 4930).

References


