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Protolichesterinic acid enhances doxorubicin-induced apoptosis in HeLa cells *in vitro*

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ABSTRACT

Aim: The aim of this study was to investigate the effect of protolichesterinic acid, a lichen secondary metabolite, on anti-proliferative activity of doxorubicin in three human cancer cell lines, HeLa, SH-SY5Y and K562 cells.
Main methods: The data obtained from MTT assays, performed on cells treated with protolichesterinic acid and doxorubicin alone and in combination, were analysed by the median-effect method as proposed by Chou and Talalay and the Bliss independence model. Apoptosis rate was evaluated by fluorescence microscopy, caspase-3, 8 and 9 activities were detected by spectrofluorimetric analysis and protein expression of Bim, Bid, Bax and Mcl-2 was analysed by Western blotting. The interaction of protolichesterinic acid with thioesterase domain of human fatty acid synthase (hFAS) was investigated by a molecular docking study.
Key findings: The *in vitro* activity of doxorubicin against HeLa cancer cell line, but not against SH-SY5Y and K562 cells, was synergically increased by protolichesterinic acid. The increased cytotoxicity caused by protolichesterinic acid in HeLa cells was due to a pro-apoptotic effect and was associated to caspase-3, 8 and 9 activation. The simultaneous treatment for 24 h with protolichesterinic acid plus doxorubicin caused an increase of Bim protein expression and the appearance of cleaved form of Bid protein. The molecular modelling analysis showed that protolichesterinic acid seemed to behave as a competitive inhibitor of hFAS.
Significance: These results suggest that protolichesterinic acid could be envisaged as an useful tool against certain types of tumor cells in combination with anticancer drugs.

Chemical compounds

Doxorubicin (PubChem CID: 31703)
Protolichesterinic acid (PubChem CID: 468953)

1. Introduction

Many different biological properties, such as anti-inflammatory, antimicrobial, antioxidant, cytotoxic and antiproliferative activities, have been associated to lichen compounds [1–4]. In the recent years, the interest in the pharmaceutical potential of lichen metabolite [5,6] is increasing even if the biological activity and therapeutical potential of relatively few compounds have been deeply investigated.

The lichen compound (+)-protolichesterinic acid, an aliphatic α-methylene butyrolactone, showed antiproliferative activity on mitogen-stimulated lymphocytes and on several human cancer cell lines [7–10], but its mechanism of action is not yet fully elucidated. Protolichesterinic acid was able to induce apoptosis through a caspase-dependent pathway in androgen-sensitive (LNCaP) and androgen-insensitive (DU-145) human prostate cancer cells [9] and in HeLa cells [10]. In *in vitro* assays, protolichesterinic acid showed inhibitory action against 5-lipoxygenase from porcine leucocytes [11], platelet-type 12-lipoxygenase [12], human DNA ligase I [13] and DNA polymerase of HIV-1 reverse transcriptase [14]. In the last years, many studies demonstrated that some natural products exhibited a synergic antitumor effect with conventional chemotherapeutic agents [15–18]. Phytochemical agents have pleiotropic effects with several intracellular targets [19,20]; they can contribute to kill tumor cells, overcome drug resistance and allow to use lower doses of anticancer drugs and, consequently, reduce toxicity, due to their simultaneous influence on different signalling pathways.

Doxorubicin is a widely used anthracyclin anticancer agent that is classified as topoisomerase II poison. This drug, upon DNA intercalation, blocks DNA replication and transcription [21]. Moreover its cytotoxic

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effect has been associated with the production of reactive oxygen species (ROS) [22]. Unfortunately doxorubicin may affect the growth not only of cancer cells but even of normal cells in the body.

Several adverse side effects are associated to doxorubicin treatment in patients, the most severe of which is cardiac toxicity [21].

In the present study, the effect of protolichesterinic acid on anti-proliferative activity of doxorubicin in three human cancer cell lines, HeLa (cervix adenocarcinoma), SH-SY5Y (neuroblastoma) and K562 (chronic myeloid leukemia) cells was investigated.

2. Materials and methods

2.1. Materials

Acridine orange, dithiotreitol (DTT), dimethyl sulfoxide (DMSO), ethidium bromide, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], Triton X-100, were purchased from Sigma Chemical Co. Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, foetal bovine serum were from Euroclone. Fluorogenic caspase substrates, acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin (Ac-DEVD-AMC), acetyl-Ile-Glu-Thr-Asp-aminotrifluoromethylcoumarin (Ac-IETD-AFC), acetyl-Leu-Glu-His-Asp-aminomethylcoumarin (Ac-LEHD-AMC) and doxorubicin were from Alexis Biochemicals. Polyvinylidene difluoride (PVDF) was from Bio-Rad. Anti-Bid, -Bim antibodies were from Cell Signalling Technology; anti-Mcl-1, -Bax, -actin, -β-tubulin antibodies were from Santa Cruz Biotechnology, Inc. All other chemicals were reagent grade. Stock solutions of protolichesterinic acid and doxorubicin were prepared in DMSO and stored in the dark at −20 °C.

2.2. Protolichesterinic acid

(+)-Protolichesterinic acid (Fig. 1) was isolated and purified from *Cornicularia aculeata* (Schreb.) Ach., collected in Ardley Cove, King George Island, Shetland del Sur, Antarctica, as previously described [23]. The degree of purity, determined by thin layer chromatography and ^{1}H NMR analysis, was higher than 98%.

2.3. Cell culture

The HeLa, SH-SY5Y and K562 cell lines was obtained from the American Type Culture Collection. The HeLa and SH-SY5Y cells were grown in DMEM medium, while the K562 cells were cultured in RPMI 1640 medium. The media supplemented with 10% heat-inactivated foetal bovine serum and containing 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM glutamine at 37 °C in a humidified 5% CO_2 atmosphere. Cell viability was determined by trypan blue exclusion assay.

2.4. Cytotoxicity assay

The effects of the two chemical agents on cell viability were evaluated *in vitro* using the MTT colorimetric method to quantify the cellular metabolic efficiency [24]. Exponentially growing cells were seeded in 96-well plates and, after 24 h of growth, were treated with various concentrations of protolichesterinic acid and doxorubicin either alone and in combination for 48 h. Negative controls received the same amount of DMSO. At the end of treatment, MTT reagent was added to each well at a concentration of 0.5 mg/mL and the cells were incubated at 37 °C for further 3 h. The MTT-formazan crystals were solubilized by addition of 100 μL of acidified isopropanol (0.04 M HCl in isopropanol). The absorbance at 570 nm was determined in a microplate reader (Biorad, Model 550).

The percentage of cell survival was obtained comparing the absorbance of treated groups with that of untreated cells, the viability of which is taken as 100%.

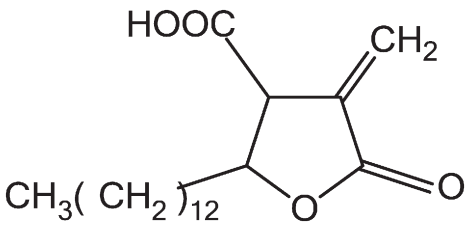

Fig. 1. Chemical structure of (+)-protolichesterinic acid.

2.5. Apoptosis assay

Analysis of nuclear morphology was assessed by double acridine orange and ethidium bromide staining. Cells were washed with PBS and were stained with a mixture, containing 100 μg/mL acridine orange and 100 μg/mL ethidium bromide in PBS. After staining, cell suspension was immediately examined under a fluorescence microscope [25]. Early apoptotic cells showed green condensed and fragmented chromatin. Late apoptotic cells showed orange clumped and fragmented nuclei.

2.6. Caspase activity

Control and treated cells were washed and lysed in cold extraction buffer (50 mM Tris–HCl, pH 7.4, 10 mM EGTA, 1 mM EDTA, 10 mM DTT, 1% (v/v) Triton X-100) for 30 min on ice. The lysates were centrifuged at 15,000 $\times g$ for 15 min at 4 °C and the supernatants were collected.

Samples (60 μg of protein) were incubated in reaction buffer with 20 μM fluorogenic peptide substrates, Ac-IETD-AFC (caspase-8) and Ac-LEHD-AMC (caspase-9) for 1 h, Ac-DEVD-AMC (caspase-3) for 30 min, at 37 °C [26]. Fluorescence was measured on a Perkin-Elmer LS-50B spectrofluorometer, setting excitation at 400 nm and emission at 505 nm for caspase-8 activity and setting excitation at 380 nm and emission at 460 nm for caspase-3 and 9 activities.

2.7. Western blot analysis

After treatments, cells were harvested, washed with PBS and solubilized in a lysis buffer (10 mM Hepes, pH 7.2, 5 mM $MgCl_2$, 142 mM KCl, 0.2% (v/v) Nonidet P-40, 1 mM EDTA and a suitable cocktail of protease inhibitors) at 4 °C for 30 min. For each sample, 60 μg of proteins were resolved by a SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride (PVDF) membranes and probed with monoclonal anti-Bid, anti-Bim, anti-Mcl-1and anti-tubulin antibodies, and polyclonal anti-Bax and anti-actin antibodies for 1 h at room temperature. Then, the membranes were incubated with the appropriate peroxidase-conjugated secondary IgG antibodies. The blots were visualized using an enhanced chemiluminescent detection system (Thermo Scientific, Rockford, USA) and the intensity of each bands were quantified by ImageJ software and normalized with actin or tubulin levels.

2.8. Evaluation of protolichesterinic acid and doxorubicin interaction

In order to determine the effects of the interaction between protolichesterinic acid and doxorubicin in HeLa, SH-SY5Y and K562 cells, data from MTT assays were analysed by the surface response

Table 1
In vitro interaction between protolichesterinic acid and doxorubicin determined by the ΔE model.

Cell line	ΔE model		
	ΣSYN	ΣANT	INT
HeLa	425.04	−37.62	Synergism
K562	0	−31.29	Additive
SHSY-5Y	0	−32.27	Additive