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

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A B S T R A C T

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 spectrophotometric 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.

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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-SYSY (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. Dubcco'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-aminotri fluoromethylcoumarin (Ac-IETD-AMC), 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, -ß-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 1H NMR analysis, was higher than 98%.

2.3. Cell culture

The HeLa, SH-SYSY and K562 cell lines was obtained from the American Type Culture Collection. The HeLa and SH-SYSY 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 µg/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine at 37 °C in a humidified 5% CO2 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%.

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 EDTA, 1 mM DTT, 1% (v/v) Triton X-100) for 30 min on ice. The lysates were centrifuged at 15,000 × 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 Heps, pH 7.2, 5 mM MgCl2, 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-1 and 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-SYSY and K562 cells, data from MTT assays were analysed by the surface response model.

### Table 1

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

<table>
<thead>
<tr>
<th>Cell line</th>
<th>ΔE model</th>
<th>ΣSYN</th>
<th>ΣANT</th>
<th>INT</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>425.04</td>
<td>−37.62</td>
<td></td>
<td>Synergism</td>
</tr>
<tr>
<td>K562</td>
<td>0</td>
<td>−31.29</td>
<td></td>
<td>Additive</td>
</tr>
<tr>
<td>SHSY-5Y</td>
<td>0</td>
<td>−32.27</td>
<td></td>
<td>Additive</td>
</tr>
</tbody>
</table>
Fig. 2. The three-dimensional plot of the difference between the predicted percentage of growth and the experimental percentage of growth of HeLa (A), K562 (B) and SH-SY5Y (C) cells treated with protolichesterinic acid and doxorubicin combinations based on the ΔE model. The data represent the mean of three independent experiments.
method based on the Bliss independence (BI) theory [27] and by the median-effect method as proposed by Chou and Talalay [28], using the chequerboard assay method. Each experiment has been done at least in triplicate.

2.8.1. Bliss independence-based model

The combined effects of the drugs can be ascertained by using the Bliss independence-based model by measuring the effect of the individual drugs compared with those obtained experimentally. The BI theory is described by the equation: \( E = E_A \times E_B \), where \( E \) is the calculated percentage of growth based on the theoretical non-interactive combination of drug A and B, and \( E_A \) and \( E_B \) are the experimental percentages of growth of each drug acting alone.

The interaction can be visualized by plotting the difference (\( \Delta E \)) between the experimental dose–response surface and the experimental surface in a xyz-plot [29,30]. All point-by-point \( \Delta E \) values calculated are plotted on the z-axis.

Any point below the horizontal plane (negative values) are classified as antagonism, any point above (positive values) are classified as synergism. If the sum of positive values is higher than negative values it is defined as synergism (\( \Sigma SYN \)), otherwise it is possible to define Bliss antagonism (\( \Sigma ANT \)). Interactions \( < 100\% \) were considered weak, interactions between both 100% and 200% were considered moderate, while interaction >200% were considered strong [31].

2.8.2. Chou-Talalay method of the median-effect principle

The Chou-Talalay method for drug combination is based on the median-effect principle, which provides the theoretical basis for the combination index (CI) calculated as:

\[
CI = \frac{\sum (D_A \times D_B)}{D_A + D_B - D_A \times D_B}
\]

For a two drug assay CI is the sum of the ratio between the doses of the drugs in combination, \( D_1 \) and \( D_2 \), and the two drugs alone, \( D_{1A} \) and \( D_{2B} \), able to affect a fraction \( x \) (fraction affected, \( Fa \)). This method allows the quantitative determination of drug interactions by the combination index (CI). CI < 1 = 1, and > 1 indicates synergism, additive effect and antagonism, respectively.

2.9. Molecular modelling

Docking of protolichesterinic acid was based on the crystal structure of thioesterase domain (TE) of human fatty acid synthase (hFAS) from Protein Data Bank (1XKT7.PDB). Hydrogens were added and Amber charges calculated for the protein and for protolichesterinic acid. Water molecules were removed. Docking has been done imposing flexibility for the only catalytic pocket residues. The in silico analysis has been performed by using Autodock4 (version 4.2.6).

2.10. Statistical analysis

Data are reported as means ± SD. Statistical differences were calculated using the Student’s t-test. Results were considered statistically significant at \( p \) value < 0.05.

3. Results

3.1. Protolichesterinic acid and doxorubicin synergically inhibit HeLa cell proliferation

A MTT assay has been performed to study the effects of protolichesterinic acid and doxorubicin in combinational treatments on cell proliferations. Cells were treated for 48 h with increasing concentrations of drugs, both alone and in combination; from 2.5 to 80 \( \mu M \) of protolichesterinic acid and from 0.05 to 12.8 \( \mu M \) of doxorubicin for HeLa cells and from 0.25 to 80 \( \mu M \) of protolichesterinic acid and from 0.025 to 1.6 \( \mu M \) of doxorubicin for K562 and SH-SYSY cells. Then, cell survival, compared with untreated controls, was evaluated using the MTT assay.

The interaction between doxorubicin and protolichesterinic acid was evaluated by models based on the Bliss independence theory (\( \Delta E \) model) and on the Chou-Talalay method.

\( \Delta E \) model interpretation, showing a \( \Sigma SYN \) value of 425.04 for 46 combinations and \( \Sigma ANT \) value of –37.62 for 8 combinations, showed that protolichesterinic acid and doxorubicin exerted synergic effects on HeLa cell viability inhibition, while pure additivity has been demonstrated for K562 and SH-SYSY (Table 1). In Fig. 2 the \( \Delta E \) response surfaces (\( E_\text{predicted} - E_\text{measured} \)) are reported for each cellular line.

The combined effect of protolichesterinic acid and doxorubicin was also analysed using the median-effect equation of Chou and Talalay, expressed as combination index, which has been calculated for each cell line. The most advantageous combinations have been selected on the base of the following criteria: the \( Fa \) (fraction affected) value for each drug alone approximatively \( \leq 0.5 \) followed by the highest \( Fa \) value for the drug combination and the lowest CI value. For each cellular line, three possible combinations have been selected (Table 2). Even if some CI values for K562 and SH-SYSY indicate a mild synergic interaction (\( CI < 1 \)), this result was not strengthened by \( \Delta E \) model. Only for HeLa cells, both \( \Delta E \) model and combination index method assessed a synergic interaction between protolichesterinic acid and doxorubicin. For HeLa cells obtained from median-effect analysis of Chou–Talalay. Each circle represents the CI value from a specific combination treatment. CI > 1 indicates antagonist effect; CI = 1 indicates additive effect; CI < 1 indicates synergistic effect; CI ≤ 0.5 indicates strong synergistic effect. The symbol ♦ indicates the CI value from the drugs combination that was further studied.
cells the lowest CI values were calculated as 0.1715, corresponding to a doxorubicin/protolichesterinic acid combination of 1.6 μM/40 μM. However, in order to deeply explore the effect of the combination of the two drugs, the authors decided to investigate the combination 1.6 μM and 20 μM of doxorubicin and protolichesterinic acid, respectively. Although the CI (0.2378) is higher than the previously mentioned combination, the lowest concentration of protolichesterinic acid (20 μM) makes sure to operate in a “safe area”, where the effect of protolichesterinic acid alone is far from to be too high. The Fa-CI plot for HeLa cells is reported in Fig. 3.

HeLa cell growth was inhibited by doxorubicin and protolichesterinic acid alone in dose-dependent manner, but protolichesterinic acid was able to cause significant reduction of cell proliferation only at concentrations higher than 20 μM (Fig. 4A and B). However, as shown in Fig. 4B, a significant increase of growth inhibition was induced by combinational treatment with doxorubicin and 20 and 40 μM protolichesterinic acid.

3.2. Protolichesterinic acid enhanced doxorubicin-induced apoptosis in HeLa cells

Based on the reported results, the combination of protolichesterinic acid at 20 μM and doxorubicin at 1.6 μM was investigated to determine whether enhanced cytotoxicity by protolichesterinic acid involved apoptosis in HeLa cells.

Cell viability was also evaluated by trypan blue exclusion test after 14 and 24 h of exposure to protolichesterinic acid and doxorubicin alone or in combination. As shown in Fig. 5A, protolichesterinic acid, 20 μM concentration significantly enhanced cytotoxicity of doxorubicin decreasing the viability from 87.2% to 66% after 24 h of incubation.

The analysis of nuclear morphology showed that the combination of the two drugs results in a significant increasing of the number of apoptotic cells (22% and 45% after 14 and 24 h of incubation, respectively) as compared to doxorubicin alone (8.4% and 24.1% after 14 and 24 h of incubation, respectively) (Fig. 5B).

3.3. Caspase-3, 8 and 9 activation induced by protolichesterinic acid and doxorubicin treatment

In order to confirm the results of cell apoptosis assay and to evaluate whether the induction of apoptosis by combined treatment with protolichesterinic acid and doxorubicin induced caspases activation, the activities of caspase-3, 8 and 9 in cell lysates were measured.

In the first 14 h of treatment with protolichesterinic acid and doxorubicin alone no significant variation in the activities of all the three caspases was observed. When HeLa cells were treated with both compounds in combination, the activity of caspase-3 and caspase-9 significantly increased of about 4- and 2-fold, respectively, as compared with untreated cells (Figs. 6A and C). After 24 h doxorubicin alone induced activation of all the three caspases, but the

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**Fig. 4.** (A) Effect of protolichesterinic acid treatment for 48 h on HeLa cell survival. (B) Combinational treatment of HeLa cells with 20 and 40 μM protolichesterinic acid (Prot) and increasing concentrations of doxorubicin (Dox) for 48 h. Cell survival was determined by the MTT assay. The results represent the mean ± SD of three independent experiments. *Data are significantly different from doxorubicin alone-treated cells (p < 0.05).*
simultaneous exposure to protolichesterinic acid caused a higher (about 2-fold) enzymatic activity of caspase-3 and caspase-8 (Figs. 6A and B). Therefore, the induction of caspase-3 activity in HeLa cells treated with combination of protolichesterinic acid and doxorubicin was formerly associated to activation of caspase-9 and later to activation of both caspase-8 and 9.

3.4. Modulating effects of protolichesterinic acid on the expression of Bcl-2 family proteins in doxorubicin-treated HeLa cells

To further investigate the mechanism of synergistic effect among protolichesterinic acid and doxorubicin, the expression of Bcl-2 family proteins, such as pro-apoptotic Bim, Bid, Bax and anti-apoptotic Mcl-1, were evaluated by Western blot analysis. The level of protein expression in HeLa cells was determined after 24 h treatment with protolichesterinic acid and doxorubicin alone or in combination. Exposure to 20 μM protolichesterinic acid alone did not affect the expression of Bax and Bid proteins, whereas it induced only a fairly increasing of Bim expression as compared with untreated cells (Figs. 7 and 8). Even if exposure to protolichesterinic acid caused a sensibilization to apoptosis induced by doxorubicin in HeLa cells, this lichen metabolite caused an up-regulation of anti-apoptotic Mcl-1 protein (Fig. 7A, B). Nevertheless, this up-regulation was inhibited when cells were simultaneously exposed to doxorubicin. Protolichesterinic acid significantly increased expression of Bim protein (23 kDa) in doxorubicin-treated cells. The expression of Bax protein was up-regulated at a similar level by either doxorubicin alone or in combination with protolichesterinic acid (Fig. 7A, B). Moreover the decrease of full length (22 kDa) and the appearance of cleaved form (15 kDa) of Bid protein were observed (Fig. 8).

3.5. Molecular modelling

With the aim to investigate the possible mechanism of action of protolichesterinic acid, a molecular modelling has been performed. For instance, protolichesterinic acid is structurally related to the human fatty acid synthase (hFAS) inhibitor C75, which is an irreversible inhibitor of the thioesterase domain (TE) of the hFAS [32]. As previously described by molecular docking study [33], the hydrophobic moiety of C75 locates inside the distal hydrophobic pocket of TE, while the hydrophilic group is interacting with the catalytic triad, Ser2308, His2481, and Asp2338. The molecular docking study showed that protolichesterinic acid stands following the same orientation of C75, with the oxygen of the carbonyl group interacting with His2481 (N-O distance 3.19 Å) and the acyl moiety buried into the hydrophobic pocket (Fig. 9).
4. Discussion

The combination of anticancer drugs with low toxicity and multitargeting phytochemicals is believed to improve the chemotherapeutic efficacy, allowing reduction of drug dosage, resistance and toxic side effects [28]. It has been also proposed that therapies with two or more combined substances might increase the therapeutic efficacy rather than the traditional sequential approaches [34,35].

This study explores the combinational anticancer activity of protolichesterinic acid and the widely used anthracycline anticancer drug, doxorubicin. Doxorubicin antitumor biochemical action includes interaction with multiple molecular targets, such as inhibition of topoisomerase I and II, formation of free radicals and DNA adducts, binding to DNA-associated enzymes; all these events are able to produce cytotoxic effects. Doxorubicin induces apoptosis modulating the Bcl-2/Bax ratio and activating different caspases. It also promotes apoptosis and necrosis in healthy tissue causing toxicity in the brain, liver, kidney and heart [36].

In this work, the in vitro activity of doxorubicin against HeLa cancer cell line, but not against SH-SYSY and K562 cells, was synergistically increased by protolichesterinic acid, a lichen metabolite. Protolichesterinic acid, mainly at concentrations of 20 and 40 μM, increases the antiproliferative/cytotoxic activity of doxorubicin in HeLa cells. Only few studies have demonstrated that lichen metabolites exhibited a synergic antitumor effect with other chemotherapeutic drugs. Recently, a chemosensibilization was observed in SK-BR-3 human breast cancer cell line, but not in T-47D cells, treated with lapatinib, a HER2 active drug, in combination with protolichesterinic acid [37]. In SK-BR-3 cells synergic effect of protolichesterinic acid was associated to down-regulation of fatty acid synthase with secondary effects on HER2 expression and signalling [37]. Enhanced sensitivity to temozolomide by co-treatment with lobarstin, a metabolite occurring from the Antarctic lichen Stereocaulon alpinum, was described in human glioblastoma T98G cells and was attributed to reduced DNA repair [38].

The mechanism of the synergistic interaction between doxorubicin and protolichesterinic acid was further investigated by analysing the apoptosis pathway. At this purposes, a concentration of 20 μM protolichesterinic acid, which does not affect cell viability, but whose synergic effect is exerted with doxorubicin on HeLa cell proliferation inhibition, was used. The increased cytotoxicity caused by protolichesterinic acid is due to a pro-apoptotic effect associated to a
decreased cell survival. The mechanism of the combined treatment seems to be caspase-dependent involving both the extrinsic and intrinsic pathways of the caspase cascade in HeLa cells. Doxorubicin alone activates caspase-8, caspase-9 and consequently caspase-3 after 24 h of treatment, whereas in combination with protolichesterinic acid an earlier caspase-9 and 3 activation, already after 14 h of treatment and before caspase-8 activation, is observed.

The extrinsic pathway, with the caspase-8 activation, is subsequently triggered to the intrinsic one. The involvement of extrinsic pathway, rather than to be due to death receptor binding, might be the result of proteolytic cleavage of caspase-8 by caspase-3 already active; for instance, it may contribute to amplify the extent of apoptosis. Modulation of Bcl-2 family protein expression confirms the responsibility of both apoptotic pathways. Bcl-2 family proteins play an important role in the regulation of apoptotic cascade and seems related to cancer resistance to conventional chemotherapy. It was observed a significant up-regulation of Bim, a well known apoptosis promoting member of the BH3-only Bcl-2 subfamily. Bim plays an important role in Bax/Bak-mediated cytochrome c release and apoptosis; it can bind Bcl-2 or Bcl-XL and prevent their antiapoptotic function [39]. Both doxorubicin and protolichesterinic acid individually seem to contribute, in additive manner, to the Bim over-expression.

HeLa cells exposed to both drugs simultaneously show truncated form of pro-apoptotic Bid protein which is able to activate pro-apoptotic proteins, such as Bax and Bad, and to lead to mitochondrial damage. No cleavage of Bim protein was detected upon treatment with protolichesterinic acid and doxorubicin alone. Then, after 24 h of combinational treatment, apoptotic signals are amplified by simultaneous mitochondrial involvement through caspase-8 dependent cleavage of Bid to t-Bid and already active caspase-9.

Mcl-1 has been shown to carry resistance to apoptosis induced by a number of different treatments in vitro. [40]. Nevertheless, the Mcl-1 up-regulation induced by protolichesterinic acid may not play an important role in doxorubicin-induced HeLa cells apoptosis; by contrast, the caspase-3-mediated cleavage may contribute to rapid decrease of Mcl-1 protein levels in co-treated cells [41].

The molecular modelling of protolichesterinic acid into TE domain of hFAS carried out on the basis of C75, shows that this lichen secondary metabolite seems to behave as a competitive inhibitor of hFAS. The antineoplastic properties of C75 are well known as well as the potential of hFAS as therapeutic target in cancer [42].

According to the molecular modelling data, it can be likely that protolichesterinic acid acts as inhibitor of the same target of C75. Further investigations will be carried on in order to clarify the real mechanism of action of protolichesterinic acid.

5. Conclusions
On the whole, our results suggest that protolichesterinic acid, by enhancing doxorubicin-induced apoptosis, can be envisaged as an useful tool against specific types of tumor cells in combination with anticancer drugs thus allowing the use of concentrations lower than those usually employed in conventional chemotherapy. This possibility appears particularly interesting when complementary therapies are required in order to maximize the antitumor activity and minimize the toxic side effects.

Conflict of interest
There was no conflict of interest.

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