Physodic acid sensitizes LNCaP prostate cancer cells to TRAIL-induced apoptosis

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ABSTRACT

In spite of the extensive research for developing new therapies, prostate cancer is still one of the major human diseases with poor prognosis and high mortality. Therefore, with the aim of identifying novel agents with antigrowth and pro-apoptotic activity on prostate cancer cells, in the present study, we evaluated the effect of lichen secondary metabolite physodic acid on cell growth in human prostate cancer cells. In addition, we tested the apoptotic activity of physodic acid on TRAIL-resistant LNCaP cells in combination with TRAIL. The cell viability was measured using MTT assay. LDH release, a marker of membrane breakdown, was also measured. For the detection of apoptosis, the evaluation of DNA fragmentation and caspase-3 activity assay were employed. The expression of proteins was detected by Western blot analysis. It was observed that physodic acid showed a dose–response relationship in the range of 12.5–50 μM concentrations in LNCaP and DU-145 cells, activating an apoptotic process. In addition, physodic acid sensitizes LNCaP cells to TRAIL-induced apoptosis. The combination of physodic acid with other anti-prostate cancer therapies could be considered a promising strategy that warrants further investigations.

1. Introduction

A lichen is a symbiotic association between a fungus and algae and/or cyanobacteria, that differs from its contributors (Goga et al., 2017). In lichen associations, the fungus mostly contributes the visible part and almost totally belongs to Ascomycota (Popescu, 2016; Meiser et al., 2017; Rankovi‘c and Kosani’c, 2019; Rick, 2020). The mycobiont protects the photobiont from intense light and absorbs the nutrients, instead this last contributes in synthesizing organic nutrients (Popescu, 2016).

Secondary metabolites, produced by lichens as protection from various biological and physical challenges, are small chemically complex substances, most of them do not occur in other fungi or plants (Galun, 2019). Secondary metabolites such as usnic acid and atranorin are located in the cortex, whereas physodic acid and physodalic acid are present in the medulla (Türk et al., 2003; Nordin et al., 2007; Fehrer and Slavíkov’a-Bayerov’a, 2008; Nelsen and Gargas, 2008; Ahmed et al., 2017). The synthesis of lichen secondary metabolites is mostly by acetate polymalonate (polyketides) pathway, which is principally connected with the fungal part of lichen (Hager et al., 2008; Mohammadi et al., 2020). Many of these compounds show interesting pharmacological activities such as antibiotic, antimycobacterial, antimutagenic, antioxidant, antiviral, antipyrletic, analgesic and antitumoral properties (Boustie et al., 2011). A great number of experiments revealed that lichens are a rich source of anti-cancer compounds (Russo et al., 2008; Russo et al., 2012; Dar et al., 2021). Lichen compounds can interact with all biological structures currently identified to be responsible for tumor development (Russo et al., 2008; Russo et al., 2012; Dar et al., 2021). For example, in our previous study the depsidine physodic acid (Fig. 1) inhibited the vitality of melanoma cells, activating an apoptotic process (Cardile et al., 2017). The results obtained suggest that physodic acid could be evaluated as a cancer-focused promising drug candidate molecule.

In spite of the extensive research for developing new therapies, prostate cancer is still one of the major human diseases with poor prognosis and high mortality (Armstrong and Gao, 2021). Therefore,
finding effective anticancer drugs endowed with low toxicity to formulate new treatment strategies is important in patients with this cancer. In view of these considerations, we aimed to develop physodic acid -mediated new approaches for the treatment and prevention of prostate cancer.

Some tumor cells, including prostate cancer, are resistant to TRAIL-induced apoptosis (Ghafouri-Fard et al., 2021). Recent research reports have shown that natural chemopreventive agents have the therapeutic potential to sensitize prostate cancer cells to TRAIL (Ghafouri-Fard et al., 2021). Therefore, we also tested the apoptotic activity of physodic acid on TRAIL-resistant LNCaP cells in combination with TRAIL.

2. Materials and methods

2.1. Chemicals

All reagents were of commercial quality and were used as received. 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) and b-nicotinamide-adenine dinucleotide (NADH) were obtained from Sigma Aldrich Co (St. Louis, USA). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and all other chemicals were purchased from Sigma Aldrich Co (St. Louis, USA) and GIBCO BRL Life Technologies (Grand Island, NY, USA). Physodic acid was isolated from Hypogymnia lugubris (Pers.) Krog collected on King George Island, South Shetland Islands, Antarctic. General experimental details have been reported previously (Cardile et al., 2018). Its purity grade was 99%.

2.2. Cell culture and treatments

LNCaP cell line was grown in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml fungizone. The cells were plated at a constant density to obtain identical experimental conditions in the different tests, thus to achieve a high accuracy of the measurements. In the MTT assay the cells were plated at 6 × 10^3 cells per well for human cancer cells, and at 2 × 10^4 cells per well for normal human non-immortalised buccal fibroblast cells, in a 96-well flat-bottomed 200 μl microplate. For other tests, the cancer cells were plated at 8 × 10^5 cells (2 ml) per 35 mm culture dish. After 24h incubation at 37 °C under a humidified 5% carbon dioxide the cells were treated with different concentrations of physodic acid, and incubated for 72 h under the same conditions. In the experiments with TRAIL, LNCaP cells were treated with TRAIL (22, 44, 88 ng/ml) and/or physodic acid (50 μM) for 12 h. Stock solution of compounds was prepared in DMSO and the final concentration of this solvent was kept constant at 0.25%. Control cultures received DMSO alone.

2.2.1. MTT bioassay

MTT assay was performed as described previously (Cardile et al., 2018).

2.2.2. Lactate dehydrogenase (LDH) release

LDH activity was spectrophotometrically measured in the culture medium and in the cellular lysates at 340 nm by analyzing NADH reduction during the pyruvate-lactate transformation, as previously reported (Cardile et al., 2018).

2.2.3. Activity of Caspase-3

The activity of caspase-3 was determined by using the Caspase colorimetric assay Kit (SIGMA RBI, St. Louis, MS, USA), as previously described (Cardile et al., 2018). The total protein content was measured according to Bradford (1976).

2.2.4. DNA analysis by COMET assay

The presence of DNA fragmentation was examined by single cell gel electrophoresis (COMET assay), as previously reported (Cardile et al., 2018).

2.2.5. TUNEL assay (ApoAlert® DNA fragmentation assay)

The nuclear DNA fragmentation was evaluated by a commercial kit (ApoAlert® DNA fragmentation Assay, Clontech Laboratories, Inc.) in accordance with the manufacturer’s instructions, as previously reported (Cardile et al., 2018).

2.2.6. Western blot analysis

The expression of Bcl-2, Bax, Hsp70, NOSi, cleaved caspase-9 and cleaved caspase-3 proteins was evaluated by Western blot analysis, as previously described (Cardile et al., 2018). Bcl-2 (SAB2500154, Sigma Aldrich) (1:500 dilution), Bax (B3428, Sigma Aldrich) (1:2000 dilution), Hsp70 (4G4, sc-59,569; Santa Cruz Biotechnology) (1:300 dilution), NOSi (N-20, sc-651; Santa Cruz Biotechnology) (1:200 dilution), cleaved caspase-9 (AB3629, Sigma Aldrich) (1:500 dilution), cleaved caspase-3 (AB3623, Sigma Aldrich) (1:200 dilution), and α-tubulin (T5326; Sigma Aldrich) (1:5000 dilution) antibodies were diluted in TBST.

2.2.7. Reactive oxygen species (ROS) assay

ROS determination was performed by using a fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA), as previously described (Cardile et al., 2018). The total protein content was measured according to Bradford (1976).

2.2.8. Statistical analysis

Representative data from three independent experiments, performed
in quadruplicate, are shown and quantified, and represented as mean ± SD. Results were analyzed as previously described (Cardile et al., 2018).

3. Results

In this study physodic acid (Fig. 1) was tested in vitro for its potential human tumor cell growth inhibitory effect on LNCaP and DU-145 tumor cell lines. It was observed that this depsidone showed a clear dose-response relationship in the range of 12.5–50 μM concentrations and a similar growth inhibitory activity in DU-145 cells as compared with LNCaP cells (Fig. 2). In addition, physodic acid, in the same experimental conditions, revealed no cytotoxic effect against normal cells (Fig. 2). LDH activity in the culture medium is used as an indicator of membrane integrity, and thus a measurement of cytotoxicity. No statistically significant increase in LDH release was observed after the treatment with physodic acid also at the concentration of 50 μM (Table 1).

Caspase-3 is the major executioner caspase in the caspase cascade, therefore examined the role of activation of this protein in cell growth inhibition mediated by tested compound (Asadi et al., 2022). The activity of caspase-3 was significantly increased in cancer cells treated for 72 h with physodic acid (Fig. 3). Nuclear DNA was analyzed using the COMET assay, a method useful for detecting apoptosis (Cardile et al., 2018). Quantification of data is reported as TMOM in Fig. 4. The tail moment (TMOM) is defined as the product of the percentage of DNA in the tail of the comet and TD value, which is obtained calculating the distance between the center of mass of the comet head and the center of mass of the tail. According caspase-3 activity, the results clearly evidenced an increase in TMOM value (Fig. 4). In the present study, we also examined the expression level of four proteins (Bcl-2, Bax, NOSi, Hsp70) involved in the apoptotic process. The Bcl-2 family proteins govern decision steps that determine whether certain caspase family cell death proteases remain quiescent or become active (Basu, 2022). Therefore, it is important that we observed that treatment with physodic acid at 12.5–50 μM shifted the Bax/Bcl-2 ratio in favor of apoptosis (Fig. 5). In addition, a down-regulation of NOSi and Hsp70 proteins was observed after the exposure with the lichen compound (Fig. 6).

ROS have oncogenic features (Kohan et al., 2020), we therefore examined whether physodic acid-induced cell death may result from an elevation of ROS. We found that the DCF fluorescence increased significantly and in a concentration-dependent manner in the human cancer cells exposed to physodic acid (Fig. 7).

LNCaP cells are more refractory to TRAIL induced apoptosis than DU-145 cells (Shankar et al., 2007). Therefore, we examined in LNCaP cells after physodic and TRAIL co-treatment, the antigrowth effect (MTT assay) and apoptosis using DNA analysis (COMET assay, TUNEL assay) together with caspase-3 activity, caspase-3 and caspase –9 expression. As shown in Fig. 8A1, after 8 and 12 h physodic acid (12.5, 25, 50 μM) and TRAIL (22, 44, 88 ng/ml) no affect cancer cells. Next, we examined

Table 1
Lactate dehydrogenase (LDH) release, expressed as percentage of LDH released into the cell medium with respect to total LDH, in prostate cancer cells treated with different concentrations of physodic acid for 72 h.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>LnCaP</th>
<th>DU-145</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.15 ± 0.7</td>
<td>3.60 ± 0.7</td>
</tr>
<tr>
<td>Physodic acid 12.5 μM</td>
<td>4.13 ± 0.8</td>
<td>3.30 ± 0.4</td>
</tr>
<tr>
<td>25 μM</td>
<td>4.40 ± 0.9</td>
<td>5.50 ± 0.6</td>
</tr>
<tr>
<td>50 μM</td>
<td>4.50 ± 0.4</td>
<td>3.70 ± 0.5</td>
</tr>
</tbody>
</table>

The values are the mean ± SD of three experiments, performed in quadruplicate.
the effect on cell viability of combining physodic acid at 50 μM concentration with different concentrations of TRAIL. Physodic acid synergized with TRAIL to induce an anti-growth effect (Fig. 8A2) and apoptosis (Figs. 8B,C and 9A,B). In fact, we evidenced a high DNA fragmentation (Fig. 8B,C) after TRAIL and physodic acid co-treatment, with an increase in caspase-3 activity and caspase-3 and caspase-9 expression (Fig. 9A,B).

4. Discussion

Natural products are proving to be an advantageous source for developing novel anticancer therapies (Akkol et al., 2020; Fernández et al., 2021). In this context, secondary metabolites of lichens have great attention in preclinical cancer studies due to their biological activity, such as anti-inflammatory and anti-neoplastic effects (Ahmed et al., 2017; Ranković and Kosanić, 2019; Mohammadi et al., 2020). In particular, studies have revealed the efficacies of
various lichen acids with respect to the anticancerous property both in vivo and in vitro (Ahmed et al., 2017; Ranković and Kosanić Ranković and Kosanić, 2019; Mohammadi et al., 2020; Dar et al., 2021). Physodic acid, a depsidone derived from Hypogymnia physodes, H. lugubris and Pseudevernia furfuracea (Dar et al., 2021), has been evaluated against brain cancer, ovarian cancer, bladder cancer, breast cancer, melanoma, colorectal cancer, pancreatic cancer, and leukemia (Dar et al., 2021; Kello et al., 2021). The cytotoxicity effect of physodic acid in our previous study was investigated on A375 melanoma cancer cell line. It reduced the growth of melanoma cells inducing apoptosis, as demonstrated by the reduction of Bcl-2 levels and over-expression of Bax and caspase-3 activity. In addition, the activation of apoptotic process probably involve the down regulation of Hsp70 (Cardile et al., 2017). Herein, for the first time, we have investigated the potential therapeutic effects of physodic acid on prostate cancer cells. Our findings demonstrated that it, as other depsidones (Russo et al., 2008; Russo et al., 2012), significantly decreased the viability of LNCaP and DU-145 cell lines. These results, according previous data (Studzinska-Sroka et al., 2021), acquire more value considering that physodic acid, in the same conditions, no affecting normal cells (Fig. 2), and support the use of this natural compound as anti-prostate cancer agent.

The ability of cancer cells to elude apoptosis is considered as one of the hallmarks of cancer and is involved in the development of multi-drug resistance (MDR) (Neophytou et al., 2021). Interestingly, in our experimental conditions physodic acid was able to induce apoptosis, as suggested by an increase of caspase-3 enzyme activity (Fig. 3) and a high DNA fragmentation (Fig. 4), not correlated to LDH release (Table 1).

Proteins implicated in the intrinsic pathway of apoptosis, including the Bcl-2 superfamily, have been implicated in the development of MDR in many cancer types. Multiple studies indicated that high levels of Bcl-2...
expression correlate with severity of malignancy in cancer patients, including prostate cancer, and are associated with resistance to chemotherapy and radiation (Basu, in press). Therefore, it is an important result that the treatment with the compound physodic acid shifted the Bax/Bcl-2 ratio in favor of apoptosis (Fig. 5). Members of the Hsp70 family of molecular chaperones are over-expressed in many cancers (Hoter et al., 2019). In particular, high expression of Hsp70 is believed to be essential for the survival of many cancer cells (Hoter et al., 2019). For example, in prostate cancer cells, Hsp70 promotes the over-expression of anti-apoptotic proteins such as Bcl-2. Critically, over-expression of Hsp70 makes prostate cancer cells insensitive to radiation therapy and increases their resistance to some drugs like etoposide, doxorubicin, and cisplatin (Hoter et al., 2019). Our data reinforce the hypothesis that the reduction of Hsp70 levels, induced in prostate cancer cells by physodic acid could induce cell death by the intrinsic or mitochondrial apoptotic pathway (Fig. 6). In addition, physodic acid inhibited the expression of NOSi (Fig. 6).

ROS participate in multiple signaling cascades in cancer cells, such as those involved in survival, proliferation, angiogenesis and metastasis and are considered responsible for cancer initiation, development and progression (Kohan et al., 2020). But, ROS production, triggering cell death, is also one of the tools included in various therapeutic strategies, comprehending chemotherapy, radiotherapy and photodynamic therapy (Kohan et al., 2020). Some ROS-generating agents of natural origin prove to be useful to avoid cell chemoresistance and can sensitize them to normal chemotherapy (Kohan et al., 2020). For example, curcumin showed this effect when administered with 5-fluorouracil in colon cancer or with tamoxifen in melanoma cells (Kohan et al., 2020). Thus, it is possible that physodic acid increasing ROS levels (Fig. 7) may act similarly.

Numerous tumor cells, including prostate cancer cells, are resistant to apoptosis mediated by TRAIL (tumor necrosis factor-related apoptosis-inducing ligand). A mechanisms of TRAIL-resistance include the over expression of anti-apoptotic proteins, such as Bcl-2 which block the pro-apoptotic proteins by forming heterodimers with them. On the other hand, deficiency of Bax expression evokes TRAIL-resistance in tumor cells. Caspase activity also affects the sensibility of cancer cells to TRAIL-induced apoptosis (Ghafouri-Fard et al., 2021). Recently, different natural compounds have been reported to sensitize tumors to TRAIL-induced apoptosis (Ghafouri-Fard et al., 2021). In view of these literature data and our present results, we examined in LNCaP cells that are more refractory to TRAIL induced apoptosis than DU-145 cells (Shankar et al., 2007), whether physodic acid can be useful to enhance the therapeutic potential of TRAIL. Physodic acid synergized with TRAIL to induce an anti-growth effect (Fig. 8A2) and apoptosis (Figs. 8B,C and 9A,B). These data are important considering that TRAIL exerts pro-apoptotic effects on malignant cells without any toxic effect to normal cells (Ghafouri-Fard et al., 2021). They might suggest hope for improvement as a new chemotherapeutic path to human prostate cancer. In particular considering the medical, social and financial effects of prostate cancer treatment, as well as mortality from it, the use of the identified synergism of this unique combination, can be a significant improvement in the quality of life of prostate cancer patients that present resistance to chemotherapy and radiation.

5. Conclusions

Physodic acid has exhibited antigrowth activity in LNCaP and DU-145 cells, activating an apoptotic process and increasing ROS production. These data suggest that the combination of physodic acid with other phytochemicals, chemotherapeutic drugs or irradiation could be an approach for the treatment of prostate cancer. However, the novel finding in the present work is that this lichen compound sensitizes LNCaP prostate cancer cells to TRAIL-induced apoptosis. Therefore, the possible therapeutic applications of these promising results should be elucidated in future in vivo studies.
Author contributions


Declaration of Competing Interest

The authors declare that they have no known competing financial-interest or personal relationships that could have appeared to influence the work reported in this paper.

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