Phytomedicine 42 (2017) 83–89

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.com/locate/phymed

Original Article

Apoptosis and pro-death autophagy induced by a spirostanol saponin isolated from *Rohdea chinensis* (Baker) N. Tanaka (synonym *Tupistra chinensis* Baker) on HL-60 cells

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ARTICLE INFO

Keywords:
Spirostanol saponin
Autophagy
Apoptosis
Rohdea chinensis
Human acute promyelocytic leukemia

ABSTRACT

Background: Our previous study has revealed that the spirostanol saponins isolated from the rhizomes of *Rohdea chinensis* (Baker) N. Tanaka (synonym *Tupistra chinensis* Baker) (Convallariaceae) (a reputed folk medicine) exhibited potent antiproliferative activity. However, the underlying mechanism of purified saponins remains unclear. More studies are necessary to assess the apoptosis and autophagy activities of the saponins from *R. chinensis* and clarify their antiproliferative mechanisms.
Purpose: The present study certificated the potential antiproliferative activity and mechanism of 5*β*-spirost-25(27)-en-1*β*,3*β*-diol-1-*O*-*α*-L-rhamnopyranosyl-(1→2)- *β*-D-xylopyranosyl-3-*O*-*α*-L-rhamnopyranoside (SPD), a spirostanol saponin from *R. chinensis*, against human acute promyelocytic leukemia cells (HL-60).
Methods: The antiproliferative activity of SPD *in vitro* was evaluated by MTT assay compared with cis-dichlorodiammineplatinum (II). The autophagic activity was assessed using MDC staining and western blot, cell apoptosis inspection was detected by Annexin V-FITC/PI double staining and the mitochondrial membrane potential was detected by JC-1 fluorescence dye combined with flow cytometry. The potential mechanisms for protein levels of apoptosis and autophagy were evaluated by western blot.
Results: Treatment of HL-60 cells with SPD resulted in growth inhibition (IC_{50} value of 2.0 ± 0.2 μM, after 48 h treatment) and induction of apoptosis and autophagy. Results from Annexin V-FITC/PI double-staining assay and mitochondrial membrane potential detection showed that apoptosis was happened after SPD treatment. The regulation of caspase-3, Bax, Bcl-2, PARP following SPD treatment contributed to the induction of mitochondria-dependent apoptosis. Meanwhile, SPD induced autophagy related with Akt/mTOR/p70S6K signaling and activated of AMPK signaling pathway. Furthermore, blocking autophagy with bafilomycin A1 reduced the cytotoxicity of SPD in HL-60 cells.
Conclusion: The antiproliferative, apoptosis and pro-death autophagy activities of SPD suggested that spirostanol saponins from *R. chinensis* would be a potential cytotoxic candidate against acute promyelocytic leukemia.

Introduction

Apoptosis, defined as type I programmed cell death, plays a significant role in tumor cell proliferation and tumor treatment (Ichim and Tait, 2016). Deregulation proliferation and inhibition of apoptosis lie at the center of tumor development and cancer therapeutic intervention (Evan and Vousden, 2001). Since cancer can deregulate cell proliferation, apoptosis plays an important role in cancer therapy (Reed and Pellecchia, 2005). There are two clearly recognized pathways modulating apoptosis, mitochondrial pathway (regulated by Bcl-2 protein family) and death-receptor pathway (triggered by death receptors). In some case, the extrinsic pathway can intersect the intrinsic pathway (Youle and Strasser, 2008). Loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) and upregulations of proapoptotic proteins were related to the mitochondria-dependent apoptosis.

Autophagy is the key process for cell homeostasis, and it is believed

Abbreviations: Akt, protein kinase B; AMPK, adenosine 5′-monophosphate-activated protein kinase; Baf-A1, bafilomycin A1; FBS, fetal bovine serum; JC-1, 5,5´,6,6´-tetrachloro-1,1´,3,3´-tetraethyl benzimidazolyl carbocyanine iodide; LC3, microtubule-associated protein light chain 3; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5 -diphenyl-2H-tetrazolium bromide; MDC, Monodansylcadaverin; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; PARP, poly-ADP-ribose polymerase; PBS, phosphate buffer saline; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; p70S6K, ribosomal protein S6 kinase; 4E-BP1, eukaryotic initiation factor 4E binding protein 1

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https://doi.org/10.1016/j.phymed.2018.03.030
Received 25 December 2017; Received in revised form 2 February 2018; Accepted 15 March 2018
0944-7113/ © 2018 Published by Elsevier GmbH.

to be highly associated with cancer (Codogno and Meijer, 2005; Wullschleger et al., 2006). It is considered as a survival mechanism induced in adverse conditions to maintain cell integrity, but paradoxically, it is also involved in a particular mode of death called autophagic cell death or type II cell death. Recently, autophagy has been argued to be another form of programmed cell death (Tsujimoto and Shimizu, 2005). Phosphorylation activation of phosphatidylinositol-3-kinase (PI3K) class I and its downstream molecules AKT and mammalian target of rapamycin (mTOR) can inhibit autophagy (Manning and Cantley, 2007; Wullschleger et al., 2006). mTOR regulates autophagy by phosphorylating downstream targets p70S6k and 4E-BP1. AMPK is a crucial regulator of energy balance. Even though the AMPK-mediated inhibition of mammalian TOR (mTOR) has been fairly well established, direct evidence for its ability to induce autophagy is still lacking in mammalian cells (Kim et al., 2011; Mihaylova and Shaw, 2011).

Natural products derived from plant sources have been regarded as an immeasurable source of potential therapeutic agents. *Rohdea chinensis* Baker (syn. *Tupistra chinensis*) (Convallariaceae) is distributed mostly in Southwest China. Its dried rhizome is a reputed Chinese folk medicine to reduce carbuncles and ameliorate pharyngitis (Xiang et al., 2016c). Steroidal saponins were believed to be the main active ingredients in this plant. Total saponins of *R. chinensis* inhibited cell proliferation and induced apoptosis in human A549 cells via mitochondria-dependent apoptotic pathways (Huang et al., 2012). Meanwhile, spirostanol saponins isolated from the rhizome of *R. chinensis* displayed potent antiproliferative and anti-inflammatory activities (Xiang et al., 2016b; Xiang et al., 2016d). However, studies on the mechanism of antiproliferative activities of spirostanol saponins isolated from *R. chinensis* have not yet reported. Therefore, the objective of this study is to investigate the antiproliferative potential of a spirostanol saponin in this plant, which is important to exploit the plentiful resource of *R. chinensis*. Mechanistically, we find out that 5*β*-spirost-25(27)-en-1*β*,3*β*-diol-1-*O*-*α*-L- rhamnopyranosyl-(1→2)-*β*-D-xylopyranosyl-3-*O*-*α*-L-rhamnopyranoside (SPD, Fig. 1) (Xiang et al., 2016a), can induce apoptosis in HL-60 cells. SPD may also promote apoptosis by induction of pro-death autophagy. Thus, it is necessary to clarify the possible potential SPD-induced antiproliferative activity and mechanisms.

Materials and methods

Experimental materials

RPMI-1640 medium and fetal bovine serum (FBS) were products of Thermo Scientific (Waltham, MA, USA). Primary antibodies against polymerase (PARP), caspase-3, Bcl-2, Bax, Beclin-1, p62, LC3, phospho-Akt (Ser473), total Akt, phospho-mTOR (Ser2448), mTOR, phospho-p70S6 kinase (Ser371), total p70S6 kinase, phospho-4E-BP1 kinase (Ser65), 4E-BP1 and beta-actin were purchased from Cell Signaling Technology (Danvers, MA, USA) for western blot assay. MTT, MDC and Baf-A1 were purchased from Sigma-Aldrich (St. Louis, MO, USA). RIPA (Radio Immunoprecipitation Assay) cell lysis buffer and JC-1 mitochondrial membrane potential assay kit were purchased from Beyotime Biotechnology (Shanghai, China). Hyperfilm ECL was product of GE Healthcare Life Sciences (Uppsala, Sweden).

HL-60 cells (human acute promyelocytic leukemia cell line) were maintained in RPMI-1640 medium, supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. SPD of over 98% purity (see Supplementary material), has isolated and identified from *R. chinensis* in our research group as previously reported (Xiang et al., 2016a). SPD was dissolved in DMSO with a stock solution at a 10 mM concentration, and was freshly diluted to the desired concentrations with culture medium. The final concentration of DMSO was less than 0.05% (v/v).

Cell viability assay

Cytotoxicity of SPD was determined by MTT assay according to a previous method (Qiao et al., 2014). Briefly, cancer cells (2.5 × 10^4 per well) were seeded in each well of the 96-well plates. After 12 h stabilization, the cells were incubated with various concentrations of SPD for further 24 or 48 h. Cell viability was determined by the MTT assay and *cis*-dichlorodiammineplatinum (II) was used as positive control. Data were presented as mean ± SEM. of three independent experiments.

Annexin V-FITC/PI double-staining

To detect apoptosis in HL-60 cells after exposure to SPD, an Annexin V-FITC/PI apoptosis detection kit was used to quantify cell number in different stages of cell death (Vermes et al., 1995). HL-60 cells (2 × 10^5) were seeded in 6-well culture plates, treated with SPD at the indicated concentrations for 24 h. In separate experiments, HL-60 cells were treated with 2 μM of SPD for 0, 12, 24, 36, 48, or 72 h, respectively. Then the cells were stained with Annexin V-FITC/PI by incubation for 15 min at room temperature in the dark. The apoptotic cells were analyzed using a CytoFLEX flow cytometer (Beckman Coulter, Paris, France), and the results were analyzed using the CytExpert software program (Beckman Coulter).

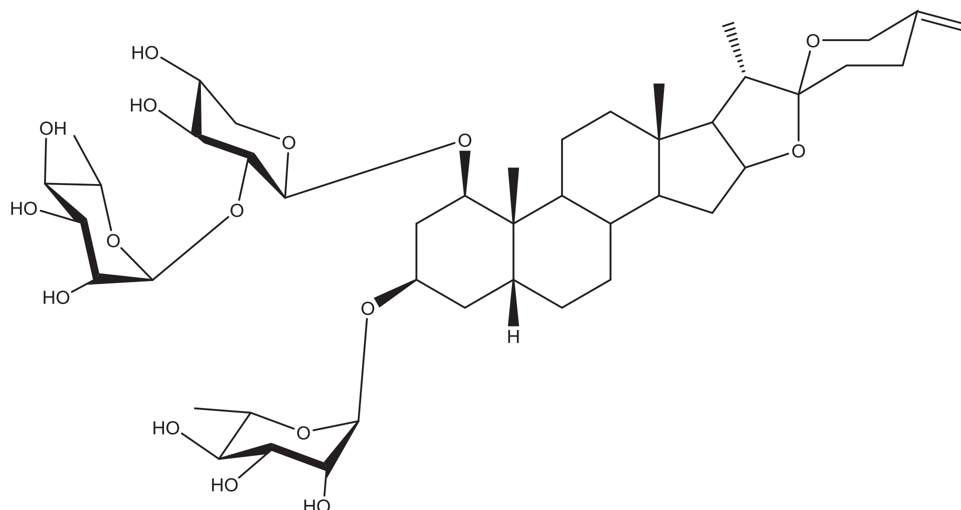

Fig. 1. Chemical structure of 5*β*-spirost-25(27)-en-1*β*,3*β*-diol-1-*O*-*α*-L- rhamnopyranosyl-(1→2)-*β*-D-xylopyranosyl-3-*O*-*α*-L-rhamnopyranoside (SPD).

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