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A eudesmane-type sesquiterpene isolated from *Pluchea odorata* (L.) Cass. combats three hallmarks of cancer cells: Unrestricted proliferation, escape from apoptosis and early metastatic outgrowth *in vitro*

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ABSTRACT

Pluchea odorata is ethno pharmaceutically used to treat inflammation-associated disorders. The dichloromethane extract (DME) was tested in the carrageenan-induced rat paw oedema assay investigating its effect on inflammation that was inhibited by 37%. Also an *in vitro* anti-neoplastic potential was reported. However, rather limited information about the bio-activity of purified compounds and their cellular mechanisms are available. Therefore, two of the most abundant eudesmanes in *P. odorata* were isolated and their anti-neoplastic and anti-intravasative activities were studied. HL-60 cells were treated with *P. odorata* compounds and metabolic activity, cell number reduction, cell cycle progression and apoptosis induction were correlated with relevant protein expression. Tumour cell intravasation through lymph endothelial monolayers was measured and potential causal mechanisms were analyzed by Western blotting. Compound PO-1 decreased the metabolic activity of HL-60 cells (IC_{50} = 8.9 μM after 72 h) and 10 μM PO-1 induced apoptosis, while PO-2 showed just weak anti-neoplastic activities at concentrations beyond 100 μM. PO-1 arrested the cell cycle in G1 and this correlated with induction of JunB expression. Independent of this mechanism 25 μM PO-1 decreased MCF-7 spheroid intravasation through the lymph endothelial barrier. Hence, PO-1 inhibits an early step of metastasis, impairs unrestricted proliferation and induces apoptosis at low micromolar concentrations. These results warrant further testing *in vivo* to challenge the potential of PO-1 as novel lead compound.

Abbreviations: CCID, circular chemo repellent-induced defect; CDK, cyclin dependent kinase; DME, dichloromethane extract (of *P. odorata*); FAK, focal adhesion kinase; HL-60, human promyelocytic leukaemia cells; HOPi, Hoechst 33258/propidium iodide double staining; HRP, horseradish peroxidase; LEC, lymph-endothelial cells T1S1; MCF-7, Michigan Cancer Foundation-7 breast cancer cells; MYPT1, myosin phosphatase 1 target subunit 1; NF-kB, nuclear factor kappa B; PO-1, (1S,2R,4aR,8aR)-[1-(acetyloxy)-1,2,3,4,4a,5,6,8a-octahydro-1,4a-dimethyl-7-(1-hydroxy-1-methylethyl)-6-oxo-2-naphthalenyl]-2,3-dimethyl-oxiranecarboxylic acid ester; PO-2, cuauthemone; WST-1, water soluble tetrazolium salt 1.

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1. Introduction

Plants supply a great variety of different secondary metabolites which are involved in defence mechanisms that provide evolutionary advantage. As unintended side effect, mankind benefits from this variety [1] and today more than 60% of all drugs used in western medicine are derivatives of natural compounds originating mostly from terrestrial plants and microbes [2]. It was estimated that only 15% of higher plants have been systematically analyzed [3] and hence, there is still a high probability to find new lead compounds particularly in plants from mega-biodiversity areas such as tropical rain forests where also the biochemical diversity is extraordinarily high [4–6]. In addition, already known plants used in traditional medicine can be tested in a new therapeutic context. As an example, the Madagascar periwinkle, *Catharanthus roseus* G. Don (Apocynaceae) was known for its anti-diabetic effects in different cultures [7] and the isolated vinca alkaloids, vincristine and vinblastine, are now used in clinical anti-cancer treatment. Another group of important plant-derived chemotherapeutics are the taxanes, which interfere, such as vinca alkaloids, with proper microtubule function, thus stopping cancer cell proliferation. The main representative of taxanes, taxol, was initially isolated from the bark of the Pacific Yew, *Taxus brevifolia* Nutt. (Taxaceae) [8], which was used by Native Americans to treat stomach and skin disorders. Hence, we focussed on a traditional medicinal plant, *Pluchea odorata* (L.) Cass., because its decoctions as well as oily and alcoholic extracts are used in Central America to treat cold, cough, flu, swelling, bruises, neuritis, rheumatic pains and inflammation [9]. Due to the fact that inflammatory as well as cancerous conditions share common intracellular signalling pathways, the dichloromethane extract (DME) of *P. odorata* was investigated regarding its potential to suppress inflammation *in vivo*, and one of its isolated main compounds to inhibit neoplastic mechanisms *in vitro*. The DME was formerly shown to exhibit an anti-inflammatory property in human umbilical vein endothelial cells (HUVECs) and anti-proliferative and pro-apoptotic activities in HL-60 promyelocytic leukaemia and MCF-7 breast cancer cells [10]. Mass spectrometry (MS) and NMR analysis identified the structures of the isolated compounds as the eudesmane-type sesquiterpenes, which were discovered before in other *Pluchea* species [11–13] but not in *P. odorata*.

Several endpoints, which are relevant anti-neoplastic indicators, were analyzed such as metabolic activity, cell number, apoptosis and intravasation of tumour cells through the lymph endothelial barrier. The expression and activity of selected proteins, which were held responsible for the DME effects [10], were here analyzed upon treatment with the isolated compounds, which allows estimating their relevance for the anti-neoplastic effects and whether also other to date unidentified compounds of the DME may be relevant for the observed pharmacological effects.

2. Materials and methods

2.1. Chemicals

Etoposide as well as monoclonal mouse ascites fluid anti-acetylated α-tubulin (6-11B-1) and α-actin (AC-15) antibodies were purchased from Sigma (St. Louis, MO, USA), monoclonal mouse α-tubulin (DM1A), polyclonal rabbit β-tubulin (H-235), paxillin (H-114) and JunB (210) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), monoclonal rabbit phospho-Cdc2 (Tyr15), polyclonal rabbit phospho-Chk2 (Thr68), Chk2, Cdc25A, Cdc2, FAK, phospho-FAK (Tyr397) and MYPT1 from Cell Signaling (Danvers, MA, USA). Polyclonal rabbit phospho-Cdc25A (Ser177) antibody was ordered from Abgent (San Diego, CA, USA) and polyclonal rabbit phospho-MYPT1 (Thr696) from Upstate (NY, USA). The secondary antibody peroxidise-conjugated anti-rabbit IgG and anti-mouse IgG were purchased from Cell Signaling (Danvers, MA, USA) and MP Biomedicals (Santa Ana, CA, USA), respectively.

2.2. Plant material

Voucher specimens (leg. G. Krupitza and R. O. Frisch, Nr. 1-2009, 08.04.2009, Herbarium W) were archived at the Museum of Natural History, Vienna, Austria. Air-dried plant material has been extracted with dichloromethane (DME) in a large scale procedure by Finzelberg GmbH & Co. KG (Andernach, Germany) as described previously [14,15].

2.3. General procedure of compound isolation

Vacuum liquid chromatography (VLC) was carried out on reversed phase C-2 silanised silica gel 60 (0.063–0.200 mm; Merck, Darmstadt, Germany) and column chromatography (CC) on silica gel 60 F_{254} (0.063–0.200 mm; Merck, Darmstadt, Germany). High performance counter current chromatography (HPCCC) experiments were conducted on a Dynamic Extractions Ltd. (Slough, UK) apparatus equipped with either a 170 ml column and a 6 ml sample loop for preparative or 32 ml column and a 2 ml sample loop for analytical runs. The spinning rate was set to 1600 rpm and a flow rate of 1.5 ml/min was utilized. As mobile phase appropriate hexan–ethylacetate–water mixtures were used.

To compare fractions, thin layer chromatography (TLC) using a $CHCl_3$–MeOH–H_2O (90:3.5:0.2 or 80:10:1) mixture as mobile phase was performed. The cytotoxic effect of fractions was evaluated using the WST-1 reagent.

2.4. Fractionation

P. odorata dichloromethane extract (DME; 62 g) was subjected to VLC (8 cm × 20 cm) on reversed phase C-2 silanised silica gel 60 using solvents with decreasing polarity [MeOH–H_2O (1:1), MeOH, MeOH–EtOAc (1:1), EtOAc, $CHCl_3$, hexane] as mobile phase. The MeOH fraction (30 g) was the most active one and thus further separated by CC (8 cm × 34 cm) using silica gel 60 F_{254} and solvents of increasing polarity (hexane, CH_2Cl_2, EtOAc, MeOH, H_2O) as mobile phase. The obtained CC fractions were combined to finally 35 fractions (B1–B35) after TLC monitoring. Fraction B12 (8.3 g) was eluted with CH_2Cl_2–EtOAc (2:1). Further fractionation of B12 by CC (8 cm × 62 cm; silica gel 60) with a $CHCl_3$–MeOH–H_2O gradient yielded 27 cumulative fractions (C1–C27). Due to the high activity (determined by WST-1 assay), fraction C10 was further separated by HPCCC. Semi-preparative fractionation [normal phase, hexane–ETOAc–MeOH–H_2O (6:5:6:5), 1.5 ml/min] was followed by purification with an analytical run (same conditions) to isolate 7 mg of PO-1 (white powder). We estimated that a total of ~100 mg PO-1 was present in 62 g DME but the majority (>90%) could not be separated from other compounds. Fraction C8 (1.5 g) contained compound PO-2 in high amounts, which was purified with silica gel 60 CC [2 cm × 80 cm, CH_2Cl_2–MeOH–H_2O (95:1.5:0.1)] to yield 440 mg of a white powder.

2.5. Identification of the isolated compounds

NMR spectra were recorded on a Bruker Avance 500 NMR spectrometer (UltraShield) using a 5 mm switchable probe (PA BBO 500SB BBFH-D-05-Z, 1H, BB = 19F and 31P–15N) with *z*-axis gradients and an automatic tuning and matching accessory (BrukerBioSpin, Rheinstetten, Germany). The resonance frequencies were 500.13 MHz for ^{1}H NMR and 125.75 MHz for ^{13}C NMR. All measurements were performed with solutions in fully deuterated chloroform at 298 K. Standard 1D and gradient-enhanced (ge)