Phytomedicine 22 (2015) 862–874

Contents lists available at ScienceDirect

Phytomedicine

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

The germacranolide sesquiterpene lactone neurolenin B of the medicinal plant *Neurolaena lobata* (L.) R.Br. ex Cass inhibits NPM/ALK-driven cell expansion and NF-κB-driven tumour intravasation

Christine Unger[a,1,*], Izabella Kiss[a,b,1], Andrea Vasas[c], Ildikó Lajter[c], Nina Kramer[a], Atanas Georgiev Atanasov[d], Chi Huu Nguyen[b], Waranya Chatuphonprasert[e,f], Stefan Brenner[e], Sigurd Krieger[b], Ruxandra McKinnon[d], Andrea Peschel[b], Renate Kain[b], Philipp Saiko[g], Thomas Szekeres[g], Lukas Kenner[b], Melanie R. Hassler[b], Rene Diaz[h], Richard Frisch[h], Verena M. Dirsch[d], Walter Jäger[e], Rainer de Martin[i], Valery N. Bochkov[j], Claus M. Passreiter[k], Barbara Peter-Vörösmarty[l], Robert M. Mader[m], Michael Grusch[l], Helmut Dolznig[a], Brigitte Kopp[d], Istvan Zupko[n], Judit Hohmann[c], Georg Krupitza[b]

[a] *Institute of Medical Genetics, Medical University of Vienna, Währinger Strasse 10, A-1090 Vienna, Austria*
[b] *Clinical Institute of Pathology, Medical University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria*
[c] *Department of Pharmacognosy, University of Szeged, Eotvos Str. 6, H-6720 Szeged, Hungary*
[d] *Department of Pharmacognosy, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria*
[e] *Department of Clinical Pharmacy and Diagnostics, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria*
[f] *Department of Preclinic, Faculty of Medicine, Mahasarakham University, Mahasarakham 44000, Thailand*
[g] *Department of Medical and Chemical Laboratory Diagnostics, Medical University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria*
[h] *Institute for Ethnobiology, Playa Diana, San José/Petén, Guatemala*
[i] *Department of Vascular Biology and Thrombosis Research, Center of Biomolecular Medicine and Pharmacology, Medical University of Vienna, Schwarzspanierstraße 17, A-1090 Vienna, Austria*
[j] *Institute of Pharmaceutical Sciences, University of Graz, Schubertstraße 1, A-8010 Graz, Austria*
[k] *Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine-University Düsseldorf, Universitätsstrasse 1, D-40225 Düsseldorf, Germany*
[l] *Department of Medicine I, Division: Institute of Cancer Research, Comprehensive Cancer Center, Medical University Vienna, Borschkegasse 8a, A-1090 Vienna, Austria*
[m] *Department of Medicine I, Comprehensive Cancer Center, Medical University Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria*
[n] *Department of Pharmacodynamics and Biopharmacy, University of Szeged, H-6720 Szeged, Hungary*

ARTICLE INFO

Article history:
Received 1 March 2015
Revised 5 June 2015
Accepted 10 June 2015

Keywords:
Neurolenins
NPM/ALK
ALCL
Intravasation
3D-compound testing
Toxicity

ABSTRACT

Background: The t(2;5)(p23;q35) chromosomal translocation results in the expression of the fusion protein NPM/ALK that when expressed in T-lymphocytes gives rise to anaplastic large cell lymphomas (ALCL). In search of new therapy options the dichloromethane extract of the ethnomedicinal plant *Neurolaena lobata* (L.) R.Br. ex Cass was shown to inhibit NPM/ALK expression.
Purpose: Therefore, we analysed whether the active principles that were recently isolated and found to inhibit inflammatory responses specifically inhibit growth of NPM/ALK+ ALCL, leukaemia and breast cancer cells, but not of normal cells, and the intravasation through the lymphendothelial barrier.
Methods: ALCL, leukaemia and breast cancer cells, and normal peripheral blood mononuclear cells (PBMCs) were treated with isolated sesquiterpene lactones and analysed for cell cycle progression, proliferation, mitochondrial activity, apoptosis, protein and mRNA expression, NF-κB and cytochrome P450 activity, 12(S)-HETE production and lymphendothelial intravasation.

Abbreviations: ALCL, anaplastic large cell lymphoma; ALOX, lipoxygenase A; CCID, circular chemorepellent induced defect; CYP, cytochrome P450; DME, dichloro methane extract; EROD, ethoxyresorufin-O-deethylase; HO/PI, Hoechst 33258/propidium iodide; LEC, lymph endothelial cell; MYPT1, myosin phosphatase 1 target subunit 1; NF-κB, nuclear factor kappa B; NPM/ALK, nucleophosmin/anaplastic lymphoma kinase, the t(2;5)(p23;q35) chromosomal translocation; PARP, poly ADP-ribose polymerase; PBMC, peripheral blood mononuclear cell; PDGF-Rβ, platelet derived growth factor receptor; p21, tumour suppressor protein 21; 3D, 3-dimensional; 12(S)-HETE, 12(S) hydroxyeicosatetraenoic acid.

* Corresponding author. Tel.: +43 1 40160 56507; fax: +43 1 40160 956501.
E-mail address: christine.unger@meduniwien.ac.at (C. Unger).
[1] Equal contribution.

http://dx.doi.org/10.1016/j.phymed.2015.06.003

Results: *In vitro* treatment of ALCL by neurolenin B suppressed NPM/ALK, JunB and PDGF-Rβ expression, inhibited the growth of ALCL cells late in M phase, and induced apoptosis via caspase 3 without compromising mitochondrial activity (as a measure of general exogenic toxicity). Moreover, neurolenin B attenuated tumour spheroid intravasation probably through inhibition of NF-κB and CYP1A1.
Conclusion: Neurolenin B specifically decreased pro-carcinogenic NPM/ALK expression in ALK+ ALCL cells and, via the inhibition of NF-kB signalling, attenuated tumour intra/extravasation into the lymphatics. Hence, neurolenin B may open new options to treat ALCL and to manage early metastatic processes to which no other therapies exist.

Introduction

Traditionally, plants have been used as remedies to treat and cure diseases including tumours. In fact, more than 60% of the currently used anti-cancer agents are derivatives of natural products (Cragg and Newman 2013). The example of vincristine (compound of *Catharanthus roseus,* the Madagaskar periwinkle) shows that a home remedy for the treatment of haemorrhage, scurvy, toothache, wounds, diabetic ulcers and hyperglycaemia (Gidding et al. 1999) was finally developed as a drug for the treatment of various cancer types. To this end we investigated the Central American plant *Neurolaena lobata* (L.) R.Br. ex Cass. (Asteraceae), which is pharmacologically active and used against ulcers, inflammatory skin disorders, malaria, ringworm, dysentery and fungal infections (Amiguet et al. 2005; Giron et al. 1991). Since inflammation and cancer often share similar cellular signalling pathways it was important that the dichloromethane fraction of the methanolic leaf extract was inhibitory in a rodent inflammation model (McKinnon et al. 2014), indicating that the extract still contained *in vivo* active principles (Butterweck and Nahrstedt 2012).

It has recently been shown that the apolar extract of *N. lobata* and the contained furanoheliangolide sesquiterpene lactone lobatin B inhibited the expression of the fusion onco-protein NPM/ALK (Kiss et al. 2015; Unger at al. 2013). This protein is generated by the t(2;5)(p23;q35) translocation and responsible for the development of ALCL (Morris et al. 1994) occurring mostly in patients at young age. The standard combination therapy (consisting of cyclophosphamide, doxorubicine, vincristine and prednisone) does not directly target the oncogenes that are involved in ALCL, i.e. NPM/ALK, JunB and PDGF-Rβ, (Laimer et al. 2012) and is known to damage DNA. This increases the likelihood of developing secondary malignancies later in life. To address this problem, the synthetic inhibitors Crizotinib and NVP-TAE-684 (Galkin et al. 2007), which selectively target ALK kinase activity, have been developed and tested in clinical trials. Here we studied germacranolide sesquiterpene lactones that interfere with NPM/ALK expression to decide about the feasibility of the development of a new drug from the isolated leads.

Methods

Plant material fine chemicals and antibodies

N. lobata (L.) R.Br. ex Cass. was collected in February 2011 in Guatemala, Departamento Petén, near the north-western shore of Lago Petén Itzá, 0.5 km NNW of San José in the area of the Chakmamantok-rock formation (16°59′16″N, 89°53′45″W) and within the botanical garden of the Institute for Ethnobiology (Unger et al., 2013). Voucher specimens (No. 813; leg. R. Diaz, det. R.O. Frisch, Chakmamantok) were deposited at the Herbarium of the Department of Pharmacognosy, University of Szeged, Szeged, Hungary. According to the Plant List data base, accepted synonyms are *N. lobata* var. *indivisa* Donn.Sm. and *N. lobata* var. *lobata*. The fresh plant material (the aerial plant parts, leaves, caulis and florescence) of *N. lobata* was air dried in Guatemala. Extraction, isolation and quantification of *N. lobata* germacranolide sesquiterpene lactones are described by McKinnon et al. (2014). *N. lobata* compounds were dissolved in DMSO (Sigma–Aldrich, St. Louis, MO, USA) as 1000-fold concentrated stock solutions. ALK-inhibitor NVP-TAE-684 (TAE-684) was from Selleckchem (Houston, TX, USA). CD246 anti-ALK protein mouse monoclonal antibody (mAB) and anti-nucleophosmin mouse mAB, were from Dako Cytomation (Glostrup, Denmark), PDGF-Rβ rabbit mAB and caspase 3 polyclonal antibody (pAB), histone H3 rabbit mAb and phospho-histone H3 rabbit pAB from Cell Signaling (Cambridge, UK). PARP-1 mouse mAB, JunB rabbit pAB, JunD rabbit pAB, c-Jun rabbit pAB, and p21 rabbit pAB, cyclin B1 rabbit pAB and GAPDH mouse mAB were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti β-actin (ascites fluid) mouse mAB was ordered from Sigma (St. Louis, MO, USA).

Cell culture

SR-786 NPM/ALK positive human ALCL (anaplastic large cell lymphoma) cells were from DSMZ (Braunschweig, Germany), CD-417 NPM/ALK positive mouse ALCL cells were isolated from *CD4*-NPM/ALK mice, HL60 (human promyelocytic leukaemia cells) were obtained from ATCC (Manassas, VA, USA). All cells were grown in RPMI 1640 medium (Life Technologies, Carlsbad, California, USA) supplemented with 10% heat inactivated fetal calf serum (FCS, Life Technologies, Carlsbad, California, USA), 1% L-glutamine (Lonza, Verviers, Belgium) and 1% antibiotics (penicillin/streptomycin (PS), Sigma–Aldrich, St. Louis, MO, USA) and maintained in a humidified atmosphere containing 5% CO_2 at 37 °C.

Isolation of peripheral blood mononuclear cells (PBMCs)

With the informed consent of the donors, PBMCs were isolated from 50 ml human peripheral blood by density-gradient centrifugation and according to standard procedures.

Proliferation assay

To determine which compounds of *N. lobata* inhibit proliferation, SR-786, CD-417, PBMC and HL60 were first counted by using a Casy cell counter (Roche Innovatis AG, Bielefeld, Germany).

Western blotting

SR-786 cells were seeded at a concentration of 2×10^5 cells/ml and CD-417 at a concentration of 1×10^6 cells/ml in 6 cm dishes. After treating cells with 3 μM of *N. lobata* compounds for the indicated times, they were harvested and lysed in RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.6, 1% Triton, 0.1% SDS, 0.5% Sodium deoxycholate) containing 1 mM phenylmethylsulfonyl (PMSF, Sigma–Aldrich, St. Louis, MO, USA) and 1 mM protease inhibitor mixture (PIM consists of 2 μg/ml leupeptin, 2 μg/ml aprotinin, 0.3 μg/ml benzamidine chloride and 10 μg/ml trypsin inhibitor, Sigma–Aldrich, St. Louis, MO, USA) followed by a short incubation of 5 min on ice. Protein concentration was determined by Bradford assay (Protein Assay Dye reagent