The dichloromethane extract of the ethnomedicinal plant *Neurolaena lobata* inhibits NPM/ALK expression which is causal for anaplastic large cell lymphomagenesis

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**Abstract.** The present study investigates extracts of *Neurolaena lobata*, an anti-protozoan ethnomedicinal plant of the Maya, regarding its anti-neoplastic properties. Firstly, extracts of increasing polarity were tested in HL-60 cells analyzing inhibition of cell proliferation and apoptosis induction. Secondly, the most active extract was further tested in anaplastic large cell lymphoma (ALCL) cell lines of human and mouse origin. The dichloromethane extract inhibited proliferation of HL-60, human and mouse ALCL cells with an IC₅₀ of ~2.5, 3.7 and 2.4 µg/ml, respectively and arrested cells in the G2/M phase. The extract induced the checkpoint kinases Chk1 and Chk2 and perturbed the orchestrated expression of the Cdc25 family of cell cycle phosphatases which was paralleled by the activation of p53, p21 and downregulation of c-Myc. Importantly, the expression of NPM/ALK and its effector JunB were drastically decreased, which correlated with the activation of caspase 3. Subsequently also platelet derived growth factor receptor β was downregulated, which was recently shown to be transcriptionally controlled by JunB synergizing with ALK in ALCL development. We show that a traditional healing plant extract downregulates various oncogenes, induces tumor suppressors, inhibits cell proliferation and triggers apoptosis of malignant cells. The discovery of the ‘Active Principle(s)’ is warranted.

**Introduction**

Natural products comprise a vast diversity of complex structures and new chemical entities, whereas synthetic libraries typically show considerably less diversity (1). Similarly to vincristine, more than 60% of currently applied anticancer drugs are derived from natural sources, i.e., plants, microorganisms and marine organisms (2). Vincristine is an alkaloid in the leaves of the Madagaskar rosy periwinkle *C. roseus*, a plant that was traditionally used to treat conditions like hemorrhage, toothache, wounds, hyperglycemia and diabetic ulcers (3). In less-developed countries, a majority of the population still relies on traditional healing plants in treating various conditions. Traditional healing plants have the advantage that they have been successfully tested over the centuries, exhibit specificity and are therefore tolerable with little side effects. This makes them a prime source for the study of new therapeutic perspectives. Therefore, natural products from high biodiversity areas such as the lowland rain forests of El Peten, Guatemala, are of particular interest. In the present work, the Maya ethno-pharmacological plant *Neurolaena lobata* was selected for investigations based on its long history as anti-protozoan medicine (4). *N. lobata* is still widely used in Guatemala and Belize to cure a variety of diseases, particularly malaria and amoebiasis, fungus, ringworm and intestinal parasites (5). More recently it is used also as oncolytic home remedy. Hypoglycemic activity of the ethanol extract was demonstrated in *vivo* (6) and a recent study reported an inhibitory effect of *N. lobata* extracts on the transfer of HIV from dendritic cells to lymphocytes in *vivo* (7). A few characteristic sesquiterpene lactones which...
were isolated of *N. lobata* (8) exhibit cytotoxic effects in GLC4 and COLO 320 tumor cell lines (9), but show only weak toxicity on brine shrimp larvae *Artemia salina* (10).

Anaplastic large cell lymphoma (ALCL) represents approximately 10-15% of childhood lymphoma, whereby the majority of them carry chromosomal aberrations involving the anaplastic lymphoma kinase (ALK) of which the t(2;5)(p23;q35) translocation, which fuses the *ALK* gene to the nucleophosmin (NPM) gene (11), represents the most frequent one (12). The resulting NPM/ALK fusion protein with constitutive tyrosine kinase activity is considered to play an essential role in the pathogenesis of ALCL through its impact on proliferation, differentiation and apoptosis (13) and malignant transformation in co-operation with other oncogenes (14). Therefore, ALK is suggested as target for therapeutic intervention. Most ALK+ ALCL patients respond with complete remission upon first-line treatment. However, high relapse rates as well as long-term effects of chemotherapy and radiation therapy have to be considered, particularly in pediatric and adolescent patients, as both treatments potentially damage normal cells which might turn into secondary malignancies within decades of life time (15-17). Combinatorial chemotherapy (CHOP: cyclophosphamide, hydroxydaunorubicin (doxorubicin), oncovin (vincristine), prednisone) is applied in the first treatment approach of ALCL patients, sometimes combined with radiotherapy. Ongoing research tries to discover a treatment directly targeting ALK (18) and an ALK inhibitor, crizotinib, is currently tested in a phase III trial (19). Here, we describe the property of the dichloromethane extract of the ethno-pharmacological plant *N. lobata*, which inhibits NPM/ALK expression and its downstream effectors and its apoptosis inducing effects on leukemia and lymphoma cells at low concentrations.

**Materials and methods**

**Plant material.** *Neurolaena lobata* was collected 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 Chakmantok-rock formation (16 59'16" N, 89 53'45" W). Voucher specimens (leg. R.O. Frisch, det. R.O. Frisch Nr. 7-2009 28. 04. 2009, Herbarium W) were archived at the Museum of Natural History, Vienna, Austria. The fresh plant material (the aerial plant parts, leaves, caulis and florescence) of *N. lobata* was stored deep-frozen until lyophilization and subsequent extraction.

**Plant extraction.** Lyophilized aerial plant parts (leaves, florescence and stipes) of *N. lobata* were powdered and consecutively extracted with petroleum ether (PE), dichloromethane (CH₂Cl₂), ethyl acetate (EA), methanol (MeOH) and water (H₂O), leading to five extracts of distinct polarity. For this, plant powder was mixed at a concentration of 1:10 (w/v) with solvent and treated in an ultra sonic bath for 10 min to break the plant cells. The extraction was performed under reflux on a waterbath for 1 h. Afterwards the content of the flask was filtered. The solid plant residue was air-dried overnight and reused for the following extraction step with the next, more polar solvent, whereas the dissolved material of the liquid phase was dried by rotary evaporation. Dried extracts were stored in a vacuum desiccator protected from light at 4°C. Extract weights obtained from serial extraction of 20.0 g lyophilized plant material with five solvents of different polarity are shown in Table I. For further use in cell culture experiments only small amounts of the gained extracts were dissolved in DMSO. To account for detrimental effects of DMSO on cell proliferation, apoptosis and cell cycle, controls were treated with the respective concentrations of DMSO used for sample treatment. Maximum concentration of DMSO was limited to 0.5% to avoid cell damage due to toxicity of DMSO.

**Cell culture.** HL-60, human promyelocytic leukemia cells, were purchased from ATCC (American Type Culture Collection). HL-60, SR-786, NPM-ALK positive human ALCL (anaplastic large cell lymphoma) cells, and CD4-417, NPM-ALK positive mouse ALCL cells were grown in RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS), 1% L-glutamine and 1% penicillin/streptomycin. Primary human lung fibroblasts (HLFs), were cultivated in DMEM medium supplemented with 10% FCS and 1% penicillin/streptomycin. Media and supplements were purchased from Life Technologies. All cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C.

**Proliferation assay.** HL-60 cells were treated with the plant extracts to discover the most anti-proliferative ones. For this, HL-60 cells were seeded in 24-well plates at a concentration of 1x10⁴ cells/ml allowing logarithmic growth within 72 h. Afterwards cells were incubated with increasing concentrations of plant extracts. The cell number was determined twice within 24 h using a KX-21 N microcell counter (Sysmex Corp., Kobe, Japan). Proliferation rates were calculated as described (20,21). Only the dichloromethane extract, which exhibited the strongest anti-neoplastic activity, was tested in other cell systems including SR-786, CD4-417 and HLF. ALCL SR-786 (human) and CD-417 (mouse) cell lines were counted using an electronic cell counter (Casy Roche). SR-786 cells were seeded in a 48-well plate at a concentration of 2x10⁵ cells/ml. Then, the cells were incubated with increasing extract concentrations for 48 h. CD4-417 cells were seeded in 48-well plates at a concentration of 1x10⁶ cells/ml and incubated with extract for 72 h.

**Alamar-blue cytotoxicity assay.** The alamar-blue assay (Invitrogen, Life Technologies) was applied to measure cytotoxicity. The active component of alamar-blue is resazurin, which is a non-toxic and cell permeable compound. Upon entering an active cell resazurin is converted to bright red fluorescent resorufin via reduction reactions of cell metabolism. The amount of fluorescence produced is proportional to the number of living cells and corresponds to the cells metabolic activity. HLF cells were seeded into 500 µl medium of a 48-well plate at a concentration providing confluence within the wells after 48 h. CD4-417 cells were seeded into 48-well plates at a concentration of 1x10⁴ cells/ml. Each well was filled with 500 µl cell suspension. Increasing concentrations of dichloromethane extract or solvent were added and after 24 and 48 h, respectively, 50 µl alamar-blue reagent was added to each well and incubated for ~90 min at 37°C until colour changed from blue to red. Afterwards the 48-well plate was placed into a multi-detection reader for fluorescence and absorbance (Bio-Tek Instrument, Inc., Vermont, USA). Plate reader software KC-4 (Bio-Tek) was used to determine absorption at 570 nm. To calculate the differences in cell viability, mean blank value (only medium) was subtracted.
and centrifuged (4˚C, 800 rpm, 5 min). The supernatant was discarded and the cell pellet was washed with cold PBS, centrifuged (4˚C, 800 rpm, 5 min), resuspended in 1 ml cold ethanol (70%), and either fixed for 30 min at 4˚C or stored at -20˚C prior further handling. After two washing steps with cold PBS, the cell pellet was resuspended in 500 µl cold PBS and transferred into a 5 ml polystyrene round bottom tube. RNAse A and propidium iodide were added to a final concentration of 50 µg/ml each and incubated for 1 h at 4˚C. The final cell number was adjusted between 0.5 and 1x10⁶ cells in 500 µl. Cells were analyzed by FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA). Cell cycle distribution was calculated with ModFid LT software (Verity Software House, Topsham, ME, USA).

**Western blotting.** CD4-417 cells were seeded in a 6-well plate at a concentration of 10⁶ cells/ml and treated with 10 µg/ml of the dichloromethane extract of *N. lobata*. SR-786 cells were seeded in a T-75 tissue culture flask at a concentration of 2x10⁵ cells/ml and incubated with 15 µg/ml dichloromethane extract. Cells (10⁶) were harvested after 4, 8 and 24 h. Additionally, Proteasome Inhibitor IV (cat. no. 539175, Merck) was added to a final concentration of 50 µM in a single experiment. In another experiment lysosome inhibitor ammonium chloride (NH₄Cl) was added at a concentration of 20 mM. HL-60 cells were seeded in T-75 tissue culture flasks at a concentration of 1.8x10⁵ cells/ml and incubated with 15 µg/ml extract. HLFs were grown in a 6-well plate and incubated with 10 and 15 µg/ml dichloromethane extract of *N. lobata*. Cells were washed twice with cold PBS and centrifuged at 1000 rpm for 5 min at 4˚C. The cell pellet was lysed in a buffer containing 150 mM NaCl, 50 mM Tris pH 8.0, 1% Triton-X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM Protease Inhibitor Cocktail (PIC), (Sigma, Schnelldorf, Germany). The lysate was centrifuged at 12000 rpm for 20 min at 4˚C. Supernatant was transferred into a 1.5-ml tube and stored at -20˚C until further analysis. Equal amounts of protein lysate were mixed with SDS (sodium dodecyl sulphate) sample buffer and loaded onto a 10% polyacrylamide gel. Proteins were separated by polyacrylamide gel electrophoresis (PAGE) at 120 V and electro-transferred onto a PVDF (polyvinylidene difluoride) membrane (Hybond, Amersham, Buckinghamshire, UK) at 95 V for 80 min. Membranes were allowed to dry for ≥30 min up to 2 h to provide fixing of the proteins to the membrane. Methanol was used to remoisten the membranes. Equal sample loading was checked by staining the membrane with Ponceau S (Sigma). After removing Ponceau S with PBS or TBS (Tris-buffered saline, pH 7.6), membranes were blocked in PBS- or TBS-milk (5% non-fat dry milk in PBS containing 0.5% Tween-20 or PBS containing 0.1% Tween-20) for 1 h. Then, membranes were washed with PBS/T (PBS containing 0.5% Tween-20) or TBS/T (TBS containing 0.1% Tween-20), changing the washing solution 4-5 times, for ≥20 min. Next, membranes were incubated with the primary antibody in blocking solution (according to the data sheet TBS-, PBS-milk or TBS-, PBS-BSA) diluted 1:500-1:1000, gently shaking at 4˚C, overnight. Thereafter, membranes were washed again with PBS/T or TBS/T and incubated with the secondary antibody (peroxidase conjugated anti-rabbit IgG or anti-mouse IgG) diluted 1:2000 in PBS- or TBS-milk at room temperature for 1 h. Chemiluminescence was developed by ECL detection kit (Amersham) and membranes were exposed to Amersham Hyperfilm.

**Table I.** *N. lobata* extract weights used for HL-60 treatment.

<table>
<thead>
<tr>
<th>Extract type</th>
<th>Used extract concentrations (µg/ml medium)</th>
<th>Corresponding dried plant weight (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether</td>
<td>5</td>
<td>113.1</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>339.2</td>
</tr>
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<td></td>
<td>30</td>
<td>678.3</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1356.7</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>5</td>
<td>168.9</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>506.8</td>
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<tr>
<td></td>
<td>30</td>
<td>1013.7</td>
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<tr>
<td></td>
<td>60</td>
<td>2027.3</td>
</tr>
<tr>
<td>Ethyl acetate</td>
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<tr>
<td></td>
<td>15</td>
<td>1024.2</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>2048.4</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>4096.8</td>
</tr>
<tr>
<td>Methanol</td>
<td>5</td>
<td>92.4</td>
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<td>277.3</td>
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<tr>
<td></td>
<td>30</td>
<td>554.6</td>
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<tr>
<td></td>
<td>60</td>
<td>1109.2</td>
</tr>
<tr>
<td>Water</td>
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<td>41.6</td>
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</tr>
<tr>
<td></td>
<td>60</td>
<td>499.0</td>
</tr>
</tbody>
</table>

from all other measurement values to take fluorescence of the medium into account. Mean value of the control samples was considered as 100% cell viability. The mean values of treated samples are described as percentage of control sample viability.

**Apoptosis assay.** Hoechst 33258 (HO) and propidium iodide (PI) double staining (both Sigma, St. Louis, MO) allows the determination of the type of death the cell is undergoing, i.e., apoptosis (early or late) or necrosis (22-24). HL-60 cells were seeded in a 24-well plate at a concentration of 1x10⁵ cells/ml and treated with increasing concentrations of the specified extracts. After 24 h of incubation, 100 µl cell suspension of each well were transferred into separate wells of a 96-well plate and Hoechst 33285 and propidium iodide were examined and photographed on a fluorescence microscope (Axiovert, Zeiss) equipped with a DAPI filter. Type and number of cell deaths was evaluated by visual examination of the images according to the morphological characteristics revealed by HOPI staining.

**Cell cycle distribution (FACS) analysis.** CD4-417 cells were seeded in a 6-well plate at a concentration of 1x10⁵ cells/ml. Then dichloromethane extract of *N. lobata* was added to a final concentration of 5, 10 and 15 µg/ml. After 24 h of incubation at 37˚C, cells were harvested, transferred into 15-ml tubes and centrifuged (4˚C, 800 rpm, 5 min). The supernatant was transferred into separate wells of a 96-well plate and Hoechst 33285 and propidium iodide were added at a final concentration of 50 µg/ml each and incubated for 1 h at 4˚C. The final cell number was adjusted between 0.5 and 1x10⁶ cells in 500 µl. Cells were analyzed by FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA). Cell cycle distribution was calculated with ModFid LT software (Verity Software House, Topsham, ME, USA).
Figure 1. Effects of different extracts of *N. lobata* on (A) proliferation and (B) apoptosis of HL-60 cells. Logarithmically growing cells were seeded at a concentration of $10^5$ cells/ml and treated with the indicated extract concentrations [(A) petroleum ether, dichloromethane, ethyl acetate, methanol, water]. Cells were counted twice within 24 h and the proliferation rate was calculated relative to solvent treated controls. (B) Cells were stained with Hoechst 33258 and propidium iodide to detect apoptotic cells using a fluorescence microscope equipped with a DAPI filter. Experiments were performed in triplicate. Error bars indicate ± SD. Asterisks indicate significant alterations in number of apoptotic cells compared to control (p<0.05).
The used antibodies were: CD246, ALK protein, monoclonal mouse, clone ALK1, code M7195 and Nucleophosmin, monoclonal mouse, clone 376, code M7305 (DakoCytomation); PDGF Receptor β (28El1) Rabbit mAb, no. 3169; Chk1 (2G1D5) Mouse mAb, no. 2360; Phospho-Chk1 (Ser345) Antibody, no. 2341; Chk2 Antibody, no. 2662; Phospho-Chk2 (Thr68) Antibody, no. 2661; Cleaved PARP (Asp214) Antibody (Mouse Specific), no. 9544; Cleaved Caspase 3 (Asp75) Antibody, no. 9661; Caspase 3 Antibody, no. 9662; Phospho-cdc2 (Tyr15) (10A11) Rabbit mAb, no. 4539; Phospho-p53 (Ser20) Antibody, no. 9287; Phospho-Akt (Ser473) (S587F1) Mouse mAb, no. 4051; Akt Antibody, no. 9272, all from (Cell Signaling); PhosphoDetect Anti-H2AX (pSer139), DR 1017 (EMD4Biosciences); p53, mouse monoclonal, cat. no. 1767 (Immunotech, Coulter Co.); PARP-1 (F-2); sc-8007, mouse monoclonal; Cdc25A (F-6): sc-7389, mouse monoclonal; Cdc25B (C-20): sc-326, rabbit polyclonal; Cdc25C (C-20): sc-327, rabbit polyclonal; Cdc2 p34 (17): sc-54, mouse monoclonal; JunB (120): sc-73, rabbit polyclonal; c-Jun (H-79): sc-1694, rabbit polyclonal; p21 (C-19): sc-397, rabbit polyclonal, all from (Santa Cruz Biotechnology, Inc.); c-Myc Ab-2 (9E10.3), no. MS-139-P1, mouse monoclonal (Thermo Fisher Scientific, Inc.); β-actin, monoclonal mouse asces fluid, clone AC-15, cat. no. A5441 (Sigma).

Quantitative RT-PCR. SR-786 cells were seeded in T-75 tissue culture flasks at a concentration of 2.5x10^3 cells/ml and incubated with 15 µg/ml dichloromethane extract of N. lobata. After 4 and 8 h cells were harvested and homogenized using Qia-shredder (Qiagen) and RNA isolated according to the instructions of RNeasy Mini Kit (Qiagen). RNA concentration was measured using a NanoDrop Fluorospectrometer (Thermo Fisher Scientific, Inc.); and RNA was stored at -80°C until further processing. First-strand cDNA synthesis from 1 µg RNA was performed using Superscript first-strand synthesis systems for RT-PCR (Invitrogen). cDNA synthesis reaction was primed using random hexamers. Desired mRNA was obtained by choosing specific primers in the PCR. NPM/ALK transcript levels in SR-786 cells were investigated by RT-PCR using TaqMan detection system. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as reference gene. For each sample, 7 µl H₂O, 10 µl TaqMan universal PCR master mix (Applied Biosystems), 1 µl primer and probe and 2 µl cDNA, or 2 µl H₂O for negative controls, were filled into a well of a 96-well optical reaction plate. In case of GAPDH amplification, the primer and probe mixture from TaqMan gene expression kit (Applied Biosystems) was used. To detect NPM/ALK transcripts, forward primer (GTG GTC TTA AGG TTG AAG TGT GGT T) and reverse primer (GCT TCC GGC GGT ACA CTA CTA A) were mixed with the probe (TGC TGT CCA CTA ATA TGC ACT GGC CCT) prior adding to the reaction mix. Final concentration of primers and probe in the sample mixtures were 0.5 and 0.25 µM, respectively. Cycle program (95°C for 10 min to activate polymerase followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min) was started on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). RT-PCR was performed in duplicates for each cDNA template and gene investigated. Negative controls, containing water instead of cDNA, confirmed the absence of RNA/DNA in all reagents applied in the assay. Comparative Ct (ΔΔCt) method (25) was used to quantify NPM/ALK expression relative to GAPDH expression using the PCR formula below. The Ct value is determined as the number of PCR cycles that is needed to reach a defined level of fluorescence and therefore newly synthesized DNA. Ratio = 2^(-ΔΔCt) ΔCt = Ct target gene (NPM-ALK) - Ct control gene (GAPDH). ΔΔCt = ΔCt drug treatment - ΔCt control sample.

Statistical analysis. The apoptosis, proliferation, toxicity, FACS and Q-PCR experiments were analyzed by t-test using GraphPad Prism version 4 (GraphPad Prim Sofware, Inc., San Diego, CA, USA). Data were considered statistically significant at p≤0.05.

Results

Anti-neoplastic activities of N. lobata extracts in HL-60 cells. To explore anti-neoplastic effects logarithmically growing HL-60 cells were treated with increasing extract concentrations (5-60 µg/ml) of the aerial parts of N. lobata and the effects on cell proliferation and apoptosis were monitored. All extracts dose-dependently inhibited proliferation whereby the dichloromethane and ethyl acetate extract exhibited the highest and the methanol and water extract the lowest activities (Fig. 1A). Therefore, the water extract was excluded from further analyses. From the remaining four extracts the dichloromethane extract induced the strongest pro-apoptotic effect (Fig. 1B) and subsequent investigations were hence continued with this extract type.

The dichloromethane extract inhibits proliferation of human and murine ALCL cells. We decided to test the N. lobata dichloromethane extract (further on simply called ‘extract’) in ALCL cells, because there is still no specific cure available for this malignancy. Human ALCL SR-786 and murine ALCL CD4-417 cells were treated with increasing concentrations of extract to analyze the inhibition of cell proliferation. The concentration inhibiting 50% of the proliferation of SR-786 cells was ~3.8 µg/ml and of CD4-417 cells ~2.5 µg/ml (Fig. 2A). In both cell lines the extract inhibited the cell cycle dose-dependently in the G2/M phase, which resulted in an accumulation of the cells in this cycle phase paralleled by a decreasing number of cells particularly in S-phase (Fig. 2B). The anti-proliferative effect of the extract was specific for the tested leukemia and lymphoma cell lines, because there was no significant toxicity observed in normal human lung fibroblasts (HLF) compared to CD4-417 cells (Fig. 2C).

N. lobata extract activates DNA damage checkpoints. In both ALCL cell lines the extract induced the phosphorylation (activation) of Chk1 and in human SR-786 cells also Chk2 activation was evident (Fig. 3A). This was consistent with the downregulation of Cdc25C, the concomitant increase of the inhibitory Cdk1 phosphorylation and with cell cycle arrest in G2/M. In contrast, the expression of Cdc25B was upregulated and Cdc25A unaffected in SR-786 cells. In murine CD4-417 cells Cdc25A became downregulated after 24 h (Fig. 3B). Conflicting signaling of Cdc25 family proteins is an alternative explanation for the observed G2/M cell cycle arrest.

N. lobata extract induces tumor suppressors and inhibits onco-genes. The induction of DNA checkpoints was paralleled by the activation of p53 indicated by its phosphorylation at serine 20 and concomitant increase of its direct transcriptional target p21.
Also c-Myc was shown to negatively regulate the transcription of p21 (26) and hence, c-Myc downregulation may have caused p21 induction. It is of note that p21 upregulation (after 4 h) precisely correlated with the downregulation of c-Myc in SR-786 cells. Also the expression of JunB was dramatically inhibited whereas c-Jun levels increased and an additional protein band with reduced electrophoretic mobility appeared on the western blotting indicating a post-translational modified c-Jun protein (Fig. 4). JunB and c-Jun are overexpressed in ALCL favoring progression (27). Taking together, the activation of p53, the induction of p21, and the inhibition of c-Myc and JunB expression certainly contributed to proliferation arrest.
N. lobata extract inhibits expression of NPM/ALK. As mentioned above, high JunB levels are common in ALCL (27) and caused by NPM/ALK (28). Hence, the decrease of JunB protein levels tempted us to analyze the effect of the extract on the expression of NPM/ALK. Treatment of SR-786 cells with the extract decreased the level of NPM/ALK with similar kinetics as JunB which is consistent with the role of NPM/ALK in the induction of JunB (Fig. 5A). Therefore, we challenged the specificity of the N. lobata extract and treated SR-786 cells with the petroleum ether extract of Critonia morifolia, which is another plant with strong anti-neoplastic properties (29). C. morifolia extract (20 µg/ml) exhibits a similar cytotoxic activity against HL-60 cells as 10 µg/ml N. lobata extract (data not shown). However, 20 µg/ml of C. morifolia extract had no effect on NPM/ALK expression in SR-786 cells (Fig. 5B). Therefore, we considered the effect of the N. lobata extract on NPM/ALK suppression as being specific. NPM/ALK was reported to permanently activate Akt via PI3K (30,31). The western blot analysis of untreated control cells showed a low constitutive Akt serine 473 phosphorylation level, which further and transiently decreased upon treatment with N. lobata extract. The downregulation of NPM/ALK correlated also with the activation of caspase 3 and the occurrence of the signature type cleavage product of PARP and an increase of γH2AX. In contrast, the expression of NPM, the 5-prime fusion partner of the t(2;5)(p23;q35) translocation was not inhibited. Next, we tested whether NPM/ALK downregulation was due to accelerated protein degradation. However, neither 20 mM ammonium chloride, which inhibits the lysosomal pathway (32) nor 50 µM Proteasome inhibitor IV reversed the reduction of NPM/ALK expression levels (Fig. 5C). Instead, the extract decreased the NPM/ALK transcript levels (Fig. 5D).

N. lobata extract inhibits expression of PDGF-Rβ. It was shown that NPM/ALK induces JunB and c-Jun (27,28). Recently
JunB and c-Jun were identified as transcriptional activators of PDGF-Rβ which synergizes with ALK in ALCL development as confirmed in an ALCL mouse model. In human ALCL PDGF-Rα takes this part. It was recently shown that imatinib and nilotinib are potent treatment options for ALCL because both inhibit PDGF-R (33). Interestingly, in established human ALCL cell lines harboring the t(2;5)(p23;q35) translocation PDGF-Rα expression disappears, whereas in murine ALCL cell lines PDGF-Rβ remains expressed in certain cell lines (33). Therefore, we tested whether extract treatment affects the expression of PDGF-Rβ in the murine CD4-417 ALCL cell line. The extract inhibited first the expression of NPM/ALK and its mediator JunB (4 h) and then the PDGF-Rβ (8 h) (Fig. 6A). While it was shown that PDGF-Rβ promotes tumor growth and progression (34,35) we demonstrate that reduced NPM/ALK expression (after 4 h) correlated with caspase 3 activation, signature type cleavage of PARP and occurrence of γH2AX, rather than with downregulation of PDGF-Rβ expression (after 8 h) (Fig. 6A). This was consistent with the observation that tyrosine kinase inhibition induced caspase-dependent apoptosis of ALK+ALCL (36). In normal HLFs PDGF-Rβ was only slightly reduced after 24 h of extract treatment and caspase 3 was not activated (Fig. 6B) indicating specificity of the extract towards malignant cells.

We wanted to know whether an ALCL standard therapy, vincristine, also downregulates NPM/ALK and PDGF-Rβ. Although vincristine dose- and time-dependently inhibited PDGF-Rβ protein expression it only marginally inhibited NPM/ALK expression (Fig. 6C). Therefore, vincristine seems to exert its anti-ALCL activity through downregulation of PDGF-Rβ and thus, targets the same protagonist as imatinib, yet by a different mechanism. In contrast, *N. lobata* extract targets mainly NPM/ALK, which further interferes with signaling downstream to

![Figure 5. Expression of NPM/ALK and apoptosis regulators upon treatment with plant extracts.](image-url)

A) SR-786 cells were incubated with 15 µg/ml N. lobata dichloromethane extract or (B) 20 µg/ml C. morifolia petroleum ether extract and harvested after the indicated times. (C) To analyse the contribution of protein degradation cells were co-treated with 20 mM ammonium acetate (left panels) to inhibit lysosome activity or with 50 µM proteasome inhibitor IV (right panels) and were harvested after 8 h. Proteins of lysed cells were electrophoretically separated and subjected to western blot analysis using the indicated antibodies. NPM/ALK was detected by using antibody against ALK, which was not expressed in these cells. Equal sample loading was confirmed by Ponceau S staining and β-actin analysis. (D) RNA was isolated, transcribed into cDNA and subjected to real-time-PCR using specific primers for NPM/ALK and GAPDH (as internal control). NPM/ALK expression was quantified using the comparative CT method. Experiments were performed in duplicates. Error bars indicate ± SD.
JunB and to PDGF-Rβ. This points towards a specific ‘active principle’ which is contained in the extract and could be used to combat ALCL. Inhibition of ALK with crizotinib and of PDGF-R with imatinib increased the survival rates in an ALCL mouse model dramatically (33). Therefore, it can be expected that the extract of \textit{N. lobata} may have a strong effect in the repression of ALCL. Furthermore, the extract must contain also other anti-neoplastic components, because HL-60 cells, which neither harbor the t(2;5)(p23;q35) translocation nor express p53 were killed by low extract concentrations.

Discussion

In search of novel cancer and lymphoma therapies we tested traditional healing plants and purified plant compounds regarding their anti-neoplastic properties (37-46). The rationale for plant selection was their ethno-pharmaceutical use against chronic inflammation or severe skin defects (4). In the present study we analysed \textit{N. lobata}, a plant which is widely used in Maya folk medicine to treat malaria (4). A panel of characteristic sesquiterpene lactones were isolated of \textit{N. lobata} such as neurolenin A-F and lobatin A-C (8) and also pyrrolizidine alkaloids (47). The plant extract and pure sesquiterpene lactones (i.e., neurolenin B), which were isolated from the dichloromethane extract, were most active against \textit{Plasmodium falciparum} and \textit{P. berghei}, whereas lobatin B was most cytotoxic in GLC4 and COLO320 tumor cell lines (9). This strongly suggests that the active principles of the dichloromethane extract used in our research were also sesquiterpene derivatives. Other drugs of this substance class, i.e., artemisinin, thapsigargin, and parthenolide, have already reached clinical trials. These compounds exhibit higher selectivity toward cancer cells then other commonly used chemotherapeutic drugs (48,49). In recent years also Maya shamans of Guatemala treat cancer patients with \textit{N. lobata} preparations. In the present study we demonstrate that the dichloromethane extract inhibits oncogenes such as c-Myc and JunB and induces the tumor suppressors p53 and p21. It blocks NPM/ALK expression at the transcriptional level and to the best of our knowledge this is the first description of a remedy with this specific property. It was shown that NPM/ALK activates PI3K and the survival promoting kinase Akt (30,31). Here, the downregulation of NPM/ALK by the \textit{N. lobata} extract correlated with the activation of caspase 3 and apoptosis. In addition, NPM/ALK induces the expression of the oncogene JunB (28) that is associated with NPM/ALK carcinogenic transformation (50). JunB overexpression is a hallmark of non-Hodgkin lymphomas such as ALCL (27) and drives the proliferation and progression of these malignancies. The kinetics of extract-mediated NPM/ALK- and JunB downregulation were similar implicating that NPM/ALK inhibition and JunB suppression were connected. Most recently it was shown that JunB and c-Jun are transcription factors of PDGF-Rβ (33). PDGF-Rβ, a receptor tyrosine kinase, which plays an important role in proliferation and differentiation (51) is overexpressed in ALCL causing the vascularization and progression of this lymphoma (33) and therefore, correlates with bad prognosis. The treatment of a terminal ALCL patient with a t(2;5)(p23;q35) translocation with imatinib, which inhibits the tyrosine kinase activity of PDGF-Rβ, caused complete remission within 14 days and this further confirms that the downstream effectors of

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**Figure 6.** Effects of \textit{N. lobata} dichloromethane extract on tyrosine kinases and apoptosis regulators. (A) CD-417 were treated with 10 µg/ml extract or (C) 1, 2.5 and 5 µM vincristine and (B) primary HLFs with 10 and 15 µg/ml extract and then cells were harvested at the indicated times, lysed and proteins subjected to electrophoretic separation and western blotting using antibodies against the indicated proteins. Equal sample loading was confirmed by Ponceau S staining and β-actin analysis.
NPM/ALK are JunB and PDGF-Rβ. Thus, the inhibition of NPM/ALK by the *N. lobata* extract most likely caused the downregulation of JunB and with some delay the decrease of PDGF-Rβ expression. The inhibition of NPM/ALK expression by *N. lobata* was specific, because the extract of *C. morifolia* or vincristine (which is used for ALCL treatment) did not affect NPM/ALK levels. *N. lobata* was toxic for malignant cells, but not for normal HLFs. In mice, oral and intra-peritoneal administration of 500 mg/kg of the water, ethanol and dichloromethane extract every 48 h for three weeks did not exhibit sub-acute toxicity and oral dosages up to 5 g/kg did not exhibit acute toxicity (52). This highlights the specificity of this *N. lobata* extract against malignant cells.

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