JPP Journal of Pharmacy and Pharmacology

JPP 2005, 57: 101–109

Received June 16, 2004
Accepted September 22, 2004
DOI 10.1211/0022357055083
ISSN 0022-3573

Apoptosis inducing activity of viscin, a lipophilic extract from *Viscum album* L.

K. Urech, J. M. Scher, K. Hostanska and H. Becker

Abstract

Detection of antiproliferative activity and bioactivity-guided fractionation of viscin, a lipophilic extract from *Viscum album* L., led to the isolation of betulinic acid, oleanolic acid and ursolic acid as active components. Viscin, betulinic acid, oleanolic acid and ursolic acid inhibited growth and induced apoptotic cell death in Molt4, K562 and U937 leukaemia cells. The growth inhibitory effect of viscin was more pronounced in Molt4 and U937 cells (IC50 (concentration that inhibited cell proliferation by 50%): 118±24 and 138±24 μg mL^{-1}) than in K562 cells (IC50: 252±37 μg mL^{-1}). Oleanolic acid was the least effective in all cell lines (7.5–45.5% inhibition at 10 μg mL^{-1}) and ursolic acid the most active in Molt4 and U937 cells (81.8 and 97.8% inhibition, respectively, at 5 μg mL^{-1}). A dose-dependent loss of membrane phospholipid asymmetry associated with apoptosis was induced in all cell lines as shown in flow cytometry by the externalization of phosphatidylserine and morphological changes in cell size and granularity. There were differences in individual cell lines' response towards the apoptosis-inducing effect of viscin, betulinic acid, oleanolic acid and ursolic acid. The triterpenoids β-amyrin, β-amyrinacetate, lupeol, lupeolacetate, β-sitosterol and stigmasterol, and the fatty acids oleic acid, linoleic acid, palmitic acid and stearic acid were also present in the lipophilic extract.

Introduction

Most observations of the pharmacological effects of *Viscum album* (mistletoe) are based on its water-soluble components (Büssing 2000). However, ethnological investigations report the medicinal application of a lipophilic fraction from *V. album* used as bird-lime since ancient times (Tubeuf 1923). This extremely sticky mass, named viscin by Macaire (1833), was in medical use in medieval times (Matthiolus 1586) and has been introduced in a commercial preparation claimed to be effective for the topical treatment of eczema, ulcers, burns and granulating wounds (Riehl 1900, Klug 1906). Pharmacological properties of mistletoe lipophilic extracts have been described in fractionation experiments identifiing antitumoral substances of *V. album*. Chloroform extracts exerted moderate inhibitory activity on tumour growth in mice but they have not been further analysed (Selawry et al 1961).

Phytochemical studies on mistletoe lipoids revealed the presence of different triterpenoids, among them betulinic acid (Krzaczec 1977), oleanolic acid (Bauer & Gerloff 1936; Wollenweber et al 2000) and ursolic acid (Krzaczek 1977). Preclinical studies have indicated that the pentacyclic triterpenes betulinic acid, oleanolic acid and ursolic acid isolated from various plants show cytotoxic and antitumour activity (Tokuda et al 1986; Fulda & Debatin 2000; Lee et al 2001; Zuco et al 2002). However, the pharmacological importance of these triterpenoids in mistletoe has never been evaluated.

Betulinic acid, oleanolic acid and ursolic acid are known to exert apoptosis-inducing activity against different malignant cells (Assefa et al 1999; Fulda & Debatin 2000; Kim et al 2000). Apoptosis is a process that regulates cell number or eliminates damaged cells. It differs morphologically and biochemically from cellular necrosis (Kerr et al 1972) and occurs in both normal and neoplastic tissues. As an active process apoptosis is characterized by condensation of nuclear chromatin, reduction of cell volume and internucleosomal DNA fragmentation (Arends et al 1990). An important

Verein für Krebsforschung, Institute Hiscia, Kirschweg 9, CH-4144 Arlesheim, Switzerland

K. Urech

Pharmakognosie und Analytische Phytochemie, Universität des Saarlandes, D-66041 Saarbrücken, Germany

J. M. Scher, H. Becker

Department of Internal Medicine, University Hospital Zürich, Rämistr. 100, CH-8091 Zürich, Switzerland

K. Hostanska

Correspondence: K. Urech, Verein für Krebsforschung, Institute Hiscia, Kirschweg 9, CH-4144 Arlesheim, Switzerland. E-mail: urech@hiscia.ch

Acknowledgement: We thank Christoph Jäggy for his help with the lay-out of the figures and Stephan Baumgartner for statistical analysis of the data.

prediction of the relevance of apoptosis to malignancy is that the rate of apoptosis versus mitosis should influence the behaviour of a tumour (Arends et al 1994).

Since the composition of the lipophilic complex of mistletoe viscin has not been analysed previously the aim of our study was to characterize the biological activity of viscin and to identify active substances by phytochemical techniques, using bioactivity-oriented fractionation. We demonstrated the antiproliferative and apoptosis-inducing activity of viscin on lymphoblastic Molt4, erythroblastic K562 and monoblastic U937 leukaemia cells and isolated and identified the active components betulinic acid, oleanolic acid and ursolic acid. Further phytochemical characterisation of viscin detected the presence of nine triterpenoids and four fatty acids.

Materials and Methods

Reagents and extracts

Triterpene acids betulinic acid, oleanolic acid and ursolic acid (each C_{30} $H_{48}O_3$; MW 456.70) were purchased from ICN Biochemicals (Aurora OH) and β-amyrin and lupeol (each $C_{30}H_{50}O$; MW 426.68) from Extrasynthèse (Geney, France). The 3-*O*-acetyl-11-keto-β-boswellic acid (AKBA; $C_{32}H_{48}O_5$; MW 512.35) isolated from *Boswellia serrata* was kindly provided by Dr H. Safayhi (Tübingen, Germany).

Viscum album ssp. *austriacum* (Wiesb.) Vollm. (Viscaceae) growing on *Pinus sylvestris* L. was collected in November in the Alpes de Hte Provence (France). Viscin was extracted from stems and leaves of this plant according to an old procedure (Tubeuf 1923): plant material (2 kg fresh) was repeatedly crushed in a mill. After kneading the wetted plant material viscin clots were collected and thoroughly washed under cold water to eliminate water-soluble material and fibres. This crude extract viscin (batch no. 5, 15.4 g) was stored at −20°C before TLC, GCMS and activity analysis.

Cell growth studies

Cytotoxicity was measured with human lymphoblastic Molt4, erythroblastic K562 and monoblastic U937 leukaemia cells from the American Type Culture Collection (Rockville, MD) and Yoshida sarcoma cells adapted for in-vitro cultivation in our laboratory (Urech et al 1995). The leukaemia cells were grown in RPMI 1640 medium (Sigma, Buchs, Switzerland) supplemented with 10% heat-inactivated fetal calf serum (FCS), and 1% penicillin–streptomycin (Sigma) in a 5% CO_2–95% air fully humidified atmosphere at 37°C. The medium for Yoshida cell growth was RPMI 1640 with 20% FCS, 1 mM pyruvate, 1 μg mL^{-1} biotin, and 1% penicillin–streptomycin. The portion of FCS was reduced to 2% in the assay medium for all cells tested. Cells were maintained in exponential growth and withdrawn for the tests at a concentration of 2–4 $\times$ 10^5 cells/mL.

Cell proliferation was indirectly measured by use of a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 (Roche Diagnostica, Rotkreuz, Switzerland) in viable cells (Ishiyama et al 1996). Cells (5×10^3 in 100 μL) were plated in triplicates in 96-well plates and kept for 1 h at 37°C before adding 10 μL tested substances. Cells were then incubated for 44 h at 37°C, and after this time 10 μL of WST-1 reagent was added per well and allowed to react for 4 h at 37°C. Absorbance at 450 nm and 650 nm (reference) was measured against a background control in a microtitre plate ELISA reader. Each experiment was repeated three times. The cell growth inhibition was calculated as follows: Inhibition (%) = 1 − (absorbance of treated/untreated control) × 100.

The concentration of substances that inhibited cell proliferation by 50% (IC50) was calculated from the dose–response curves by regression analysis. All components tested were solubilized in dimethyl sulfoxide (DMSO). The final concentration of DMSO was 0.5%.

Cell death analysis

Apoptosis was assessed using three methods. Morphological changes consistent with apoptosis were inspected by oil-immersion light microscopy of Giemsa stained cytospin slides (Del Vecchio et al 1991). Quantification of apoptosis was performed by flow cytometric analysis. To determine phosphatidylserine externalization by Annexin V adherence cultured cells (2×10^4 mL^{-1}, 48 h) were pelleted and suspended in binding buffer (1 mL; composition in mM: 10 HEPES pH 7.4, 150 NaCl, 5 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$) containing Annexin V as described previously (Vermes et al 1995; Hostanska et al 2003). Plasma membrane permeability was estimated by propidium iodide (PI; 1 μg mL^{-1}) uptake. The percentages of undamaged cells (not binding Annexin V and excluding PI) and of apoptotic cells (binding Annexin V; early apoptotic cells: PI-negative; late apoptotic cells: PI-positive) were quantified. Simultaneously, cell morphology based on light-scattering characteristics, cell size according to forward (FSC) and granularity to side scatter (SSC), was investigated. The measurements were performed on 10^4 cells analysed by use of a FACScalibur cytofluorometer (B-D, Basel, Switzerland) equipped with the CellQuest program. Vehicle-treated cells (0.5% DMSO) were always included and were not found to be affected in cell growth and apoptosis.

Phytochemical techniques

All solvents were distilled before use and removed from the extracts by rotary evaporation under reduced pressure at temperatures up to 40°C. Silica gel (LiChroprep 40–63 μm and LiChroprep 15 μm; Merck, Darmstadt, Germany) was used for vacuum liquid chromatography (VLC). Thin-layer chromatography (TLC) was performed on silica gel (Kieselgel 60 F254; Merck), reversed-phase C18 (HPTLC-plates RP18, F254; Merck). HPLC was performed on a diol modified silica gel column (Merck LiChrospher 100, diol, 250 × 4 mm^2, 5 μm, mobile phase

1.4 mL min^{-1}, RI-detection) and a reversed-phase RP18 column (Luna C18, 5 μm 250 × 4 mm^2, mobile phase 1.0 mL min^{-1}, RI-detection). CI-MS, DCI-MS data were recorded on a Finnigan MAT 90 spectrometer. ^{1}H and ^{13}C NMR spectra were performed at 500 MHz (^{1}H NMR) or 125 MHz (^{13}C NMR) on a Bruker DRX 500 (in $CDCl_3$ and C_5D_5N at 25°C).

Viscin was silylated by the method of Sweely et al (1963): viscin (0.5 g) was dissolved in chloroform (20 mL) and filtered. The solvent was evaporated in-vacuo and the residue dissolved in 4 mL pyridine. One millilitre of this solution was treated with pyridine–hexamethyldisilazane–trimethylchlorsilane (1:2:1) in a 5-mL graduated flask. The mixture was shaken vigorously for 30 s and then allowed to stand at least for 15 min at room temperature before chromatography. Gas chromatography–mass spectrometry (GCMS) was performed with a Hewlett Packard G1800A GCD system fitted with a split injection and a flame ionization detector. Separation was achieved using a HP-5 fused silica column (15 m × 0.25 mm) using He as carrier gas (1 mL min^{-1}; oven temperature 180°C for 2 min and then increased at 2°C min^{-1} up to 270°C; injector and detector temperatures: 300°C). In the chromatogram of the viscin (batch no. 5) the following peaks could be assigned: linoleic acid **11** (R_t 4.82 min), stearic acid **9** (R_t 5.37 min), lupeol **4** (32.66 min), β-amyrinacetate **2** (R_t 35.60 min), lupeolacetate **5** (R_t 37.11 min), oleanolic acid **3** (R_t 39.06 min) and betulinic acid **6** (R_t 39.67 min); numbers in bold types correspond to the chemical structures given in Figure 1. Peaks at R_t 4.10, 11.20, 11.69, 22.90, 33.18, 34.13 min could not be identified.

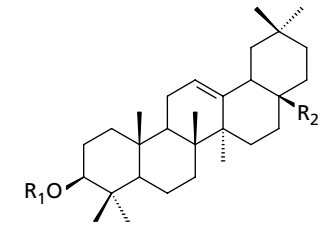
Statistical analysis

Student's two-tailed test was employed to assess the statistical significance of differences between the untreated controls and viscin-treated samples (Sachs 1992). Experiments performed in triplicate were repeated three times. IC50 values were obtained by regression analysis of data using the Microsoft Excel software. The exerted growth-inhibitory effect of the lipoid extract viscin and fractions on three leukaemia cells was analysed statistically with the Statistica/Mac software (Statsoft, USA). Mean differences were evaluated by running a one-way analysis of variance test (Sachs 1992). Post-hoc comparisons were performed with Newman–Keuls test (Horn & Vollandt 1995). $P \leq 0.05$ was considered to be statistically significant. The comparison of histograms was evaluated statistically using the Kolmogorov–Smirnov (K-S) two-samples test for overlaid histograms (Young 1977).

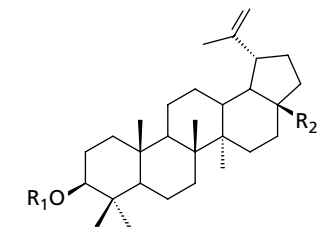
Results and Discussion

Cell-growth-inhibitory and -death-inducing activity of viscin

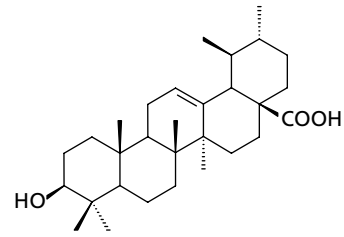
The effect of viscin on cell growth was measured in three haematological cell lines (Molt4, K562, U937). Cell



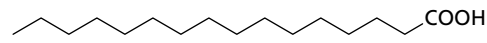
	R_1	R_2
1	H	CH_3
2	$COCH_3$	CH_3
3	H	COOH



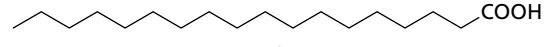
	R_1	R_2
4	H	CH_3
5	$COCH_3$	CH_3
6	H	COOH



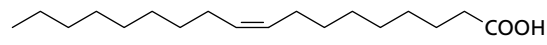
7



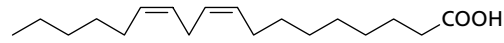
8



9



10



11

Figure 1 Structures of the pentacyclic triterpenes β-amyrin (**1**), β-amyrinacetate (**2**), oleanolic acid (**3**), lupeol (**4**), lupeolacetate (**5**), betulinic acid (**6**) and ursolic acid (**7**) and of the fatty acids palmitic acid (**8**), stearic acid (**9**), oleic acid (**10**) and linoleic acid (**11**) identified in viscin from *Viscum album*.

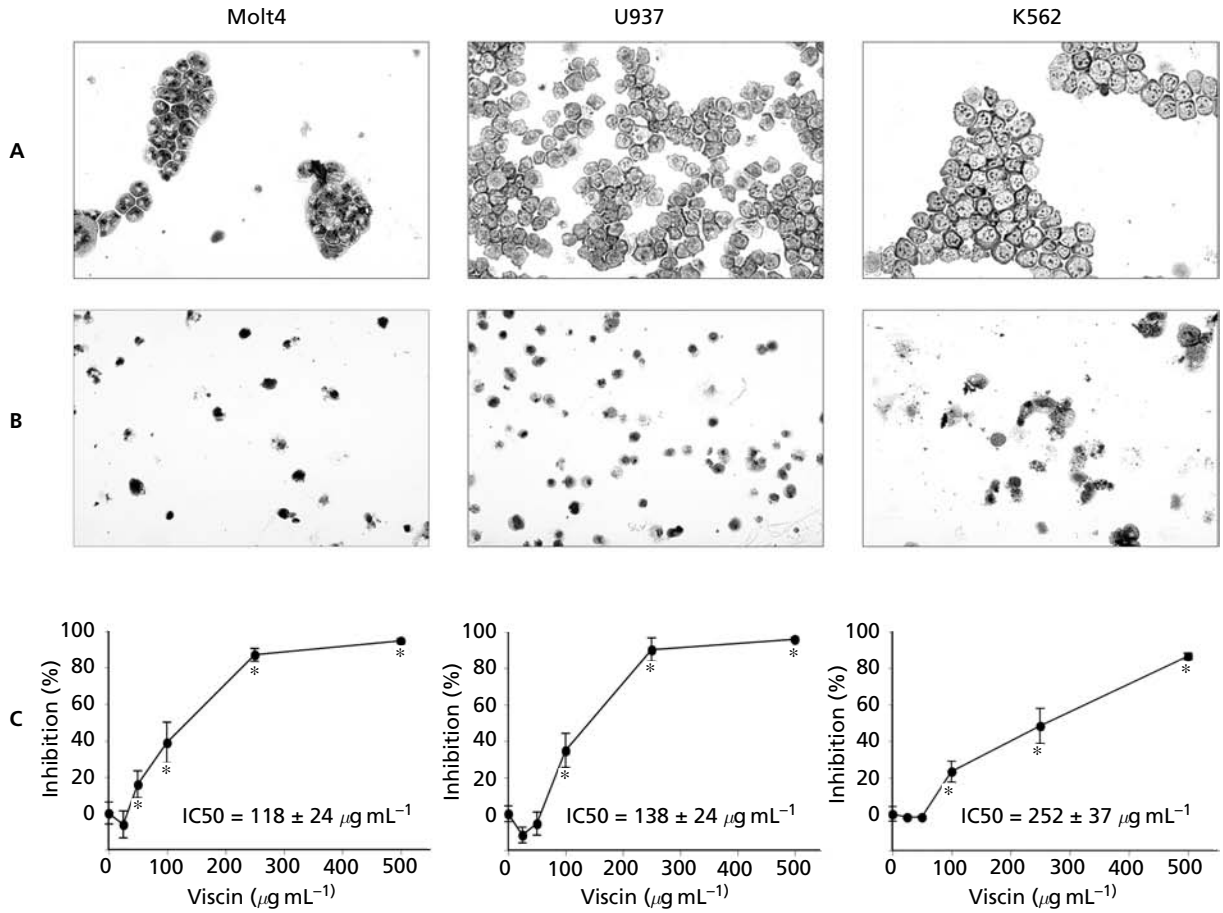


Figure 2 Antiproliferative activity of viscin in Molt4,U937 and K562 leukaemia cells. Cell growth inhibition was monitored by microscopic analysis of Giemsa-stained slides of the cultured cells (A, control cells 48 h incubated without viscin; B, cells 48 h exposed to 200 μg mL^{-1} viscin) and by measuring dose dependency of cell viability by WST-1 assay after 48 h viscin treatment (C, % inhibition of cell growth and IC50 calculated from viability data). Values are mean ± s.d. *$P < 0.05$, compared with untreated control.

growth was assessed indirectly by measuring the cleavage of tetrazolium salt WST-1 to formazan. This assay gives an indication of undisturbed mitochondrial, and also extramitochondrial, NADH- and NADPH-dependent redox enzyme systems. The effect of viscin was studied as a dose–response experiment after 48 h at concentrations of 25–500 μg mL^{-1}. All three cell lines exhibited significant viscin-induced suppression of growth above a concentration of 100 μg mL^{-1} (Figure 2). There were differences between leukaemia cell lines in susceptibility towards growth inhibition by viscin (Figure 2). The IC50 of viscin in K562 cells was about two-fold higher and differed significantly in comparison with Molt4 ($P < 0.05$) and U937 ($P < 0.05$) cells. No significant difference in sensitivity towards viscin could be detected between Molt4 and U937 cells. Viscin at a concentration of 250 μg mL^{-1} almost completely abrogated the metabolic activity of these two cell lines. As positive control the pentacyclic triterpene AKBA from the gum resin of *Boswellia serrata* was chosen, for which antiproliferative and apoptosis-inducing action in leukaemia cells has been reported (Shao et al 1998; Hostanska et al 2002). AKBA (10 μg mL^{-1}) caused 68.4%, 75.7% and 59.3% down regulation of Molt4, K562 and U937 cells growth, respectively.

A first approach to the mode of cell death by viscin was conducted by light microscopic analysis of Giemsa stained slides. Comparison with untreated controls showed that after 48 h treatment of Molt4, U937 and K562 cells with 200 μg mL^{-1}, the number of cells decreased significantly and the remaining cells reduced in size with chromatin clumping (Figure 2). These microscopic observations of morphological changes typical for apoptosis corresponded to flow cytometric cell-scatter characteristics FSC (cell size) and SSC (cell granularity) (data not shown).

To further substantiate the cell-growth-inhibitory effect of viscin, the cells were stained by Annexin V and plasma membrane permeability was estimated with PI. Annexin V is a Ca^{2+}-dependent phospholipid-binding

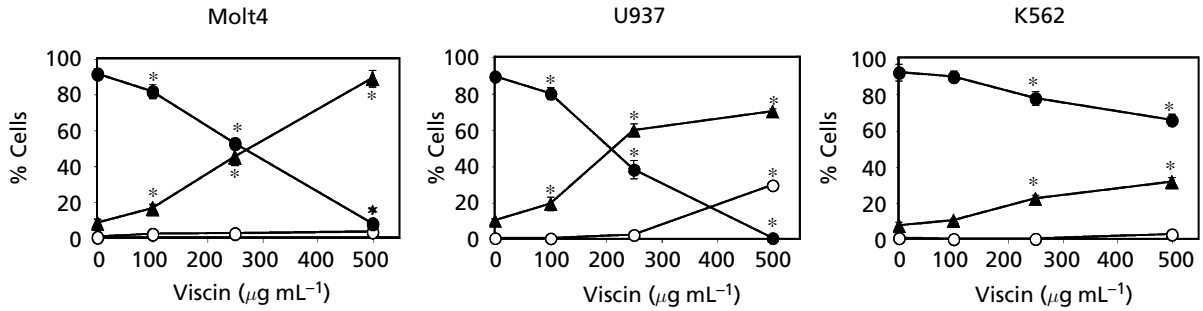


Figure 3 Dose dependency of the induction of apoptosis and necrosis in Molt4, U937 and K562 leukaemia cells in culture by viscin. The portions of apoptotic (▲), necrotic (○) and undamaged (●) cells were calculated from bivariate Annexin V/PI FACS analysis of the cells after 48 h incubation with viscin. Values are the means ± s.d. of three experiments. $*P < 0.05$, compared with untreated control.

protein with high affinity for phosphatidylserine. In viable cells, phosphatidylserine is located on the cytoplasmic surface of the cell membrane; in apoptotic cells, it is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing phosphatidylserine (Vermes et al 1995). By these staining techniques the portions of intact, apoptotic and necrotic cells after 48 h incubation with viscin were quantitatively analysed. Viscin induced apoptosis in all three cell lines in a dose-dependent manner (Figure 3). K562 appeared to be less sensitive than Molt4 or U937. Only about 30% of K562 cells exposed phosphatidylserine at the highest concentration (500 $\mu g\,mL^{-1}$) of viscin compared with about 50% of apoptotic cells for Molt4 and U937 after 250 $\mu g\,mL^{-1}$ viscin treatment (Figure 3). This difference in sensitivity corresponded to the difference in reactivity towards the antiproliferative action of viscin. However, sensitivity of all three cell lines was much higher for the growth-inhibitory effect. Reduction of cell growth can reflect either a decreased proliferation rate or an enhanced cell death by either necrosis or apoptosis or a combination of these mechanisms. Apoptotic cell death is most likely not the only mechanism involved in down regulation of cell growth by viscin. Necrosis remained at a low level (< 10%) over the whole dose range (Figure 3). Only U937 cells showed a considerable portion of necrotic cells at the highest dose of 500 $\mu g\,mL^{-1}$. Therefore the cells were possibly arrested in their cell division cycles. The active components of viscin, betulinic acid, oleanolic acid and ursolic acid, described below, are known to arrest the growth of various malignant cells predominantly in the G1 phase (Es-Saady et al 1996; Li et al 2002).

Previous reports demonstrated that mistletoe lectin induces apoptosis in various tumour cells (Janssen et al 1993; Hostanska et al 1996; Büssing & Schietzel 1999). Experiments with heat-treated viscin were conducted to exclude the presence of active lectin in viscin. Heating viscin solubilised in 100% DMSO at 80°C for 30 min did not reduce its cytotoxic activity on Molt4 cells. Therefore heat-sensitive and apoptosis-inducing mistletoe lectin did not contribute to the inhibitory effect of viscin. The viscotoxin-sensitive Yoshida sarcoma cell line was resistant to the cytotoxic activity of viscin at a concentration of 100 $\mu g\,mL^{-1}$, which inhibited Molt4 cells about 40%. As Yoshida sarcoma cells are about 22 times more sensitive towards viscotoxins than Molt4 cells (Urech et al 1995), interfering amounts of contaminating viscotoxins in viscin could also be excluded.

Fractionation and phytochemical analysis of viscin

The green gum viscin (5.0 g) was subjected to normal-phase VLC (5.0 g precoated on 20 g silica gel), fractionated in 250-mL steps from 100% hexane through EtOAc to methanol. The fractions were combined according to the accompanying TLC into 7 fractions (1–7) and tested for inhibition of proliferation of Molt4 cells. Fraction 5 showed the highest specific activity (IC50 = 25 $\mu g\,mL^{-1}$), followed by fraction 4 (IC50 = 72 $\mu g\,mL^{-1}$). The other fractions were much less active or inactive (IC50 > 500 $\mu g\,mL^{-1}$). Only fractions 2, 4 and 5 were further analysed.

Fraction 5 showed, on TLC, a non-UV-active pink spot when sprayed with anisaldehyde–H_2SO_4 (hexane–EtOAc, 7:3; R_f 0.2). Fractionation of fraction 5 was performed by diol HPLC (hexane–EtOAc–HCOOH, 89:10:1). Compound **3** (Figure 1) eluted with t_R 7 min (fraction 5.2) and a mixture of compound **6** and **7** eluted with t_R 6 min (fraction 5.3). This mixture was separated into fraction 5.3.1 (compound **7**) and fraction 5.3.2 (compound **6**) by RP18-HPLC (MeOH–H_2O–HCOOH, 89:10:1). High specific antiproliferative activity was found for the three fractions: 5.2 (oleanolic acid **3**), 5.3.1 (ursolic acid **7**) and 5.3.2 (betulinic acid **6**) (Figure 4). Their purity was determined by NMR and GCMS after silylation.

β-Amyrinacetate **2** and lupeolacetate **5** were isolated from fraction 2 by diol HPLC (hexane–EtOAc, 99.5:0.5).

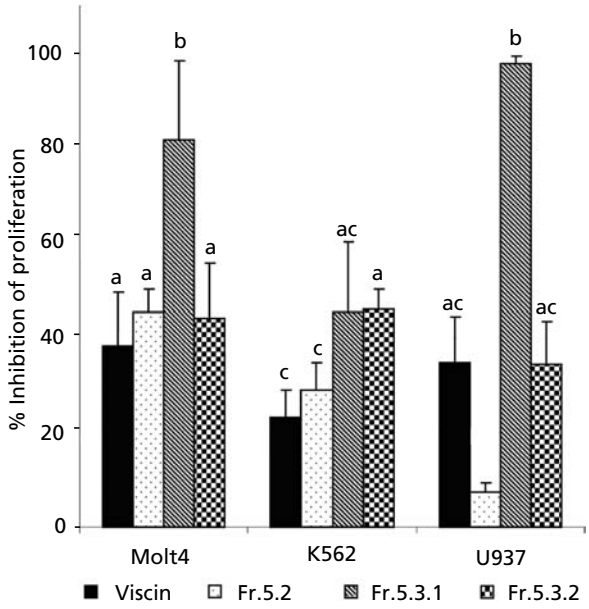


Figure 4 Antiproliferative effects of the triterpene fractions 5.2 (oleanolic acid 10 μg mL^{-1}), 5.3.1 (ursolic acid 5 μg mL^{-1}) and 5.3.2 (betulinic acid 5 μg mL^{-1}) from viscin in Molt4, K562 and U937 leukaemia cells. Reduction of the cell growth in relation to an untreated control was measured after 48 h incubation in comparison with 100 μg mL^{-1} viscin. Values are means ± s.d. of three experiments. All the values are statistically different ($P < 0.05$), except those marked with the same letters (a, b or c).

Compound **2** eluted with t_R 11 min and compound **5** with t_R 10 min.

Fraction 4 was subjected to VLC on silica gel, developed in steps from hexane to hexane–EtOAc (80:20) to yield three fractions (fractions 4.1–4.3). The two triterpenes β-amyrin **1** and lupeol **4** and the two fatty acids oleic acid **10** and stearic acid **9** were detected by GCMS in fraction 4.1. Furthermore, palmitic acid **8** was detected in fraction 4.2. The main components of fraction 4.3 were purified by diol HPLC (hexane–EtOAc, 95:5). β-Sitosterol eluted with t_R 10.5 min and linoleic acid **11** with t_R 13 min. Furthermore, stigmasterol was detected in fraction 4.3 by GCMS.

The isolated compounds were identified by comparison of their ^{1}H, ^{13}C and mass spectra with published data: β-amyrinacetate **2** (Bhattacharyya & Barros 1986), betulinic acid **6** (Idrayanto et al 1983), linoleic acid **11** (Zimmer 1997), lupeolacetate **5** (Wenkert et al 1978), oleanolic acid **3** (Zhang et al 1999), β-sitosterol and stigmasterol (De-Eknamku & Potduang 2003) and ursolic acid **7** (Lin et al 1987). The volatile compounds β-amyrin and lupeol were identified by GC comparison with the authentic compounds. Oleic acid **10**, linoleic acid **11**, palmitic acid **8** and stearic acid **9** were identified by comparison of their mass spectra (GCMS) with literature and the Wiley database. The chemical structures of the identified compounds are given in Figure 1.

Response of leukaemia cells towards the triterpenoidal fractions

The inhibitory activity in the Molt4 proliferation assay of compounds **3** (oleanolic acid), **6** (betulinic acid) and **7** (ursolic acid) isolated from mistletoe showed good agreement with corresponding commercial compounds isolated from other sources (data not shown). A comparative study showed characteristic differences between the patterns of the antiproliferative responses of the three cell lines towards the three triterpene acids (Figure 4). Fraction 5.2 (oleanolic acid) was the least active in all three cell lines and fraction 5.3.2 (ursolic acid) the most active in Molt4 and U937. Fraction 5.3.1 (betulinic acid) showed intermediate activity in Molt4 and U937 cells. In K562 cells fraction 5.3.1 (betulinic acid) and fraction 5.3.2 (ursolic acid) exerted identical activity. Reactivity depended on the structure of the triterpenes used and was individually modulated in a cell-line-specific manner. Our findings on oleanolic acid and ursolic acid are in accordance with published data on their effect on colon carcinoma HCT15 cells and leukaemia K562 and U937 (Li et al 2002; Chiang et al 2003). Both compounds share many common pharmacological properties (Liu 1995). The antiproliferative activity of betulinic acid in this study corresponds well with its activity on a panel of neoplastic cell lines reported by Zuco et al (2002).

The three structural isomeric ($C_{30}H_{48}O_3$) pentacyclic triterpenes, betulinic acid (3β-hydroxy-lup-20(29)-en-28-oic acid), oleanolic acid (3β-hydroxy-olea-12-en-28-oic acid) and ursolic acid (3β-hydroxy-urs-12-en-28-oic acid), have been well characterized for their apoptosis-inducing potency (Assefa et al 1999; Choi et al 2000; Fulda & Debatin 2000; Lauthier et al 2000; Zuco et al 2002). In this study the fractions 5.2 (oleanolic acid), 5.3.2 (ursolic acid) and 5.3.1 (betulinic acid) have been tested for their apoptotic effect by measuring the cell parameters size and granularity and by double staining of the cells with Annexin V-FITC and PI after 48 h incubation. Representative results on Molt4 cells are given in Figure 5. All three compounds showed apoptosis-inducing activity. Thus, at least part of the apoptotic potential of viscin has to be attributed to betulinic acid, oleanolic acid and ursolic acid.

Conclusion

Our study of the antiproliferative and apoptosis-inducing activity of viscin gives a first experimental basis for pharmacological evaluations of lipophilic extracts from *V. album*. Triterpene acids proved to be active constituents of viscin with antileukaemic activity in cell culture. The relevance of these mistletoe components for possible pharmacological applications can only be estimated after evaluation of the quantitative proportions in *V. album* and its extracts. Development of reliable methods for quantifying triterpenes in *V. album* is the subject of our current investigation.

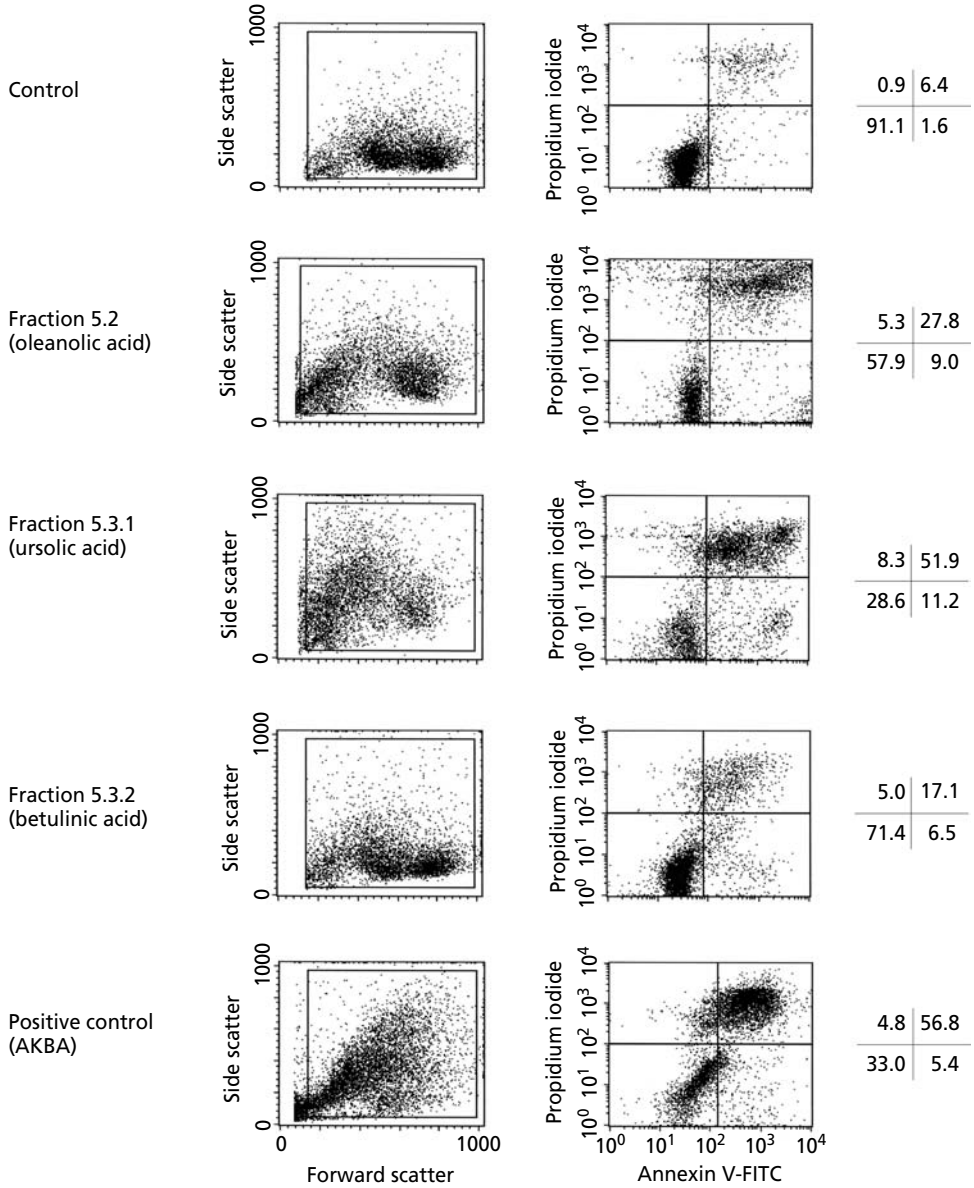


Figure 5 Flow cytometric analysis of apoptosis in Molt4 cells induced after 48 h treatment with fractions 5.2 (oleanolic acid, 20 μg mL^{-1}), 5.3.1 (betulinic acid, 10 μg mL^{-1}) and 5.3.2 (ursolic acid, 10 μg mL^{-1}) in comparison with untreated control. The two-parameter histograms in the left column blotting cell size (forward scatter) vs granularity (side scatter) show morphological changes typical for apoptosis. The AnnexinV/PI double-stained cells (see Materials and Methods) are presented in the right column of dot blots. Left lower quadrants of the dot blots represent unstained (undamaged) cells, the left upper quadrants PI-stained (necrotic) cells, the right lower quadrants Annexin V binding (early apoptotic) cells and the right upper quadrants Annexin V and PI-stained (late apoptotic) cells. The attached numbers represent the % portion of cells in the single quadrants. As positive control 5 μg mL^{-1} AKBA-treated cells were used.

References

Arends, M. J., Morris, R. G., Wyllie, A. H. (1990) Apoptosis: the role of the endonuclease. *Am. J. Pathol.* **136**: 593–608

Arends, M. J., Mc Gregor, A. H., Wyllie, A. H. (1994) Apoptosis is inversely related to necrosis and determines net growth in tumors bearing constitutively expressed myc, ras, and HPV oncogenes. *Am. J. Pathol.* **144**: 1045–1057

Assefa, H., Nimrod, A., Walker, L., Sindelar, R. (1999) Synthesis and evaluation of potential complement inhibitory semisynthetic analogues of oleanolic acid. *Bioorg. Med. Chem. Lett.* **9**: 1889–1994

Bauer, K. H., Gerloff, U. (1936) Ueber die Harzalkohole der Mistel. *Arch. Pharm.* **274**: 473–485

Bhattacharyya, J., Barros, C. B. (1986) Triterpenoids of *Cnidosculus urens*. *Phytochemistry* **25**: 274–276

Büssing, A. (2000) Biological and pharmacological properties of *Viscum album* L. In: Büssing, A. (ed.) *Mistletoe — the genus Viscum*.: Harwood Academic Publishers, Amsterdam, pp 123–182

Büssing, A., Schietzel, M. (1999) Apoptosis-inducing properties of *Viscum album* L. extracts from different host trees, correlate with their content of toxic mistletoe lectins. *Anticancer Res*. **19**: 23–28

Chiang, L. C., Chiang, W., Chang, M. Y., Ng, L. T., Lin, C. C. (2003) Antileukemic activity of selected natural products in Taiwan. *Am. J. Chin. Med*. **31**: 37–46

Choi, B. M., Park, R., Pae, H. O., Yoo, J. C., Kim, C. Y., Jun, C. D., Jung, B. H., Oh, G. S., So, H. S., Kim, Y. M., Chung, H. T. (2000) Cyclic adenosine monophosphate inhibits ursolic acid-induced apoptosis via activation of protein kinase A in human leukaemic HL-60 cells. *Pharmacol. Toxicol*. **86**: 53–58

De-Eknamku, W., Potduang, B. (2003) Biosynthesis of β-sitosterol and stigmasterol in *Croton sublyratus* proceeds via a mixed origin of isoprene units. *Phytochemistry* **62**: 389–398

Del Vecchio, M. T., Leoncini, L., Buerki, K., Kraft, R., Megha, T., Barbibi, P., Tossi, P., Cottier, H. (1991) Diffuse centrocytic and/or centroblastic malignant non-Hodgkin's lymphomas: comparison of mitotic and pyknotic (apoptotic) indices. *Int. J. Cancer* **47**: 38–43

Es-Saady, D., Simon, A., Ollier, M., Maurizis, J. C., Chulia, A. J., Delage, C. (1996) Inhibitory effect of ursolic acid on B16 proliferation through cell cycle arrest. *Cancer Lett*. **106**: 193–197

Fulda, S., Debatin, K. M. (2000) Betulinic acid induces apoptosis through a direct effect on mitochondria in neuroectodermal tumors. *Med. Pediatr. Oncol*. **35**: 616–618

Horn, M., Vollandt, R. (1995) *Multiple Tests und Auswahlverfahren*. Gustav Fischer, Stuttgart

Hostanska, K., Hajto, T., Fischer, J., Lentzen, H., Sütterlin, B., Saller, R. (1996) A natural immunity-activating plant lectin, Viscum album agglutinin-I, induces apoptosis in human lymphocytes, monocytes, monocytic THP-1 cells and murine thymocytes. *Nat. Immun*. **15**: 295–311

Hostanska, K., Daum, G., Saller, R. (2002) Cytostatic and apoptosis-inducing activity of boswellic acids toward malignant cell lines in vitro. *Anticancer Res*. **22**: 2853–2862

Hostanska, K., Reichling, J., Bommer, S., Weber, M., Saller, R. (2003) Hyperforin a constituent of St John's wort (*Hypericum perforatum* L.) extract induces apoptosis by triggering activation of caspases and with hypericin synergistically exerts cytotoxicity towards human malignant cell lines. *Eur. J. Pharm. Biopharm*. **56**: 121–132

Idrayanto, G., Voelter, W., Reinhard, E. (1983) Steroids and triterpenes in cell cultures. *Chemiker-Zeitung* **107**: 238–239

Ishiyama, M., Tominaga, H., Shiga, M., Sasamoto, K., Ohkura, Y., Ueno, K. (1996) A combined assay of cell viability and in vitro cytotoxicity with highly water soluble tetrazolium salt, neutral red and cristal violet. *Biol. Pharm. Bull*. **11**: 1518–1520

Janssen, O., Scheffler, A., Kabelitz, D. (1993) In vitro effects of mistletoe extracts and mistletoe lectins. Cytotoxicity towards tumor cells due to the induction of programmed cell death (apoptosis). *Arzneimittelforschung* **43**: 1221–1227

Kerr, J. F. R., Wyllie, A. H., Currie, A. R. (1972) Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. *Br. J. Cancer* **26**: 239–257

Kim, D. K., Baek, J. H., Kang, C. M., Yoo, M. A., Sung, J. W., Kim, D. K., Chung, H. Y., Kim, N. D., Choi, Y. H., Lee, S. H., Kim, K. W. (2000) Apoptotic activity of ursolic acid may correlate with the inhibition of initiation of DNA replication. *Int. J. Cancer* **87**: 629–636

Klug, A. (1906) Viscolan, eine neue Salbengrundlage. *Dtsch. Med. Wochenschr*. **13**: 290–291

Krzaczek, T. (1977) Pharmacobotanical research on the subspecies of *Viscum album* L. III Terpenes and sterols. *Ann. Univ. M. Curie Lublin-Polonia* **32**: 125–134

Lauthier, F., Tailet, L., Trouillas, P., Dalage, C., Simon, A. (2000) Ursolic acid triggers calcium-dependent apoptosis in human Daudi cells. *Anticancer Drugs* **11**: 737–745

Lee, S. S., Chen W. C., Huang, C. F., Su, Y. (1998) Preparation and cytotoxic effect of ceanothic acid derivatives. *J. Nat. Prod*. **61**: 1343–1347

Lee, I., Lee, J., Lee, Y. H., Leonard, J. (2001) Ursolic acid-induced changes in tumor growth, O2 consumption, and tumor interstitial fluid pressure. *Anticancer Res*. **21**: 2827–2834

Li, J., Guo, W. J., Yang, Q. Y. (2002) Effects of ursolic acid and oleanolic acid on human colon carcinoma cell line HCT15. *World J. Gastroenterol*. **8**: 493–495

Lin, C. N., Chung, M. I., Gan, K. H., Chiang, J. R. (1987) Studies on the constituents of Formosan gentianaceous plants. Part IX. Xanthones from Formosan gentianaceous plants. *Phytochemistry* **26**: 2381–2384

Liu, J. (1995) Pharmacology of oleanolic and ursolic acid. *J. Ethnopharmacol*. **49**: 57–68

Macaire M. (1833) Mémoire sur la viscine. *Société Phys. Hist. Nat*. **6**: 27–34

Matthiolus, D. P. A. (1586) Kreutterbuch, neue Auflage von Joachimum Camerarium. Frankfort a/Mayn

Riehl, G. (1900) Über Viscin und dessen therapeutische Verwendung. *Dtsch. Med. Wochenschr*. **26**: 653–655

Sachs, L. (1992) *Angewandte Statistik*. Springer, Berlin

Selawry, O., Vester, F., Mai, W., Schwartz, M. R. (1961) Zur Kenntnis der Inhaltsstoffe von *Viscum album*, II Tumorhemmende Inhaltsstoffe. *Hoppe-Seyler's Z. Physiol. Chem*. **324**: 262–281

Shao, Y., Ho, C. T., Chin, C. K., Badmaev, V., Ma, W., Huang, M. T. (1998) Inhibitory activity of boswellic acids from Boswellia serrata against leukemia HL-60 cells in culture. *Planta Med*. **64**: 328–331

Sweely, C. C., Bentley, R., Makita, M., Wells, W.W. (1963) Gas-Liquid Chromatography of trimethylsilyl derivatives of sugars and related substances. *Gas Chromatogr. Sugars* **20**: 2497–2507

Tokuda, H., Ohigashi, H., Koshimizu, K., Ito, Y. (1986) Inhibitory effects of ursolic and oleanolic acid on skin tumor promotion by 12-O-tetradecanoylphorbol-13-acetate. *Cancer Lett*. **33**: 279–285

Tubeuf, K. V. (1923) *Monografie der Mistel*. Oldenbourg, München, Berlin

Urech, K., Schaller, G., Ziska, P., Giannattasio, M. (1995) Comparative study on the cytotoxic activity of viscotoxin and mistletoe lectin on tumour cells in culture. *Phytother. Res*. **9**: 49–55

Vermes, I., Haanen, C., Steffens-Nakken, H., Reutelingsperger, C. (1995) A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J. Immunol. Methods* **184**: 39–51

Wenkert, E., Baddeley, G., Vernon, B., Ivor, R., Moreno, L. N. (1978) Carbon-13 nuclear magnetic resonance spectroscopy of naturally-occurring substances. LVII. Triterpenes related to lupane and hopane. *Organic Magnetic Resonance* **11**: 337–343

Wollenweber, E., Wieland, A., Haas. K. (2000) Epicuticular waxes and flavonol aglycones of the European mistletoe, *Viscum album* L. *Z. Naturforsch*. **55**: 314–317

Young, I. T. (1977) Proof without prejudice: use of the Kolmogorov-Smirnov test for the analysis of histograms from flow systems and other sources. *J. Histochem. Cytochem*. **25**: 935–941

Zimmer, M. (1997) *Die chinesische Maulwurfsgrille* Gryllotalpa africana *Beauvois und die saarländische Maulwurfsgrille* Gryllotalpa gryllotalpa *Linne in der Wundheilkunde*. PhD thesis, Saarbrücken, Germany

Zhang, Z., Koike, K., Jia, Z., Nikaido, T., Guo, D., Zheng, J. (1999) Four new triterpenoidal saponins acylated with one monoterpenic acid from *Gleditsia sinensis*. *J. Nat. Prod.* **62**: 740–745

Zuco, V., Supino, R., Righetti, S. C., Cleris, L., Marchesi, E., Gambacorti-Passerini, C., Formelli, F. (2002) Selective cytotoxicity of betulinic acid on tumor cell lines, but not on normal cells. *Cancer Lett*. **175**: 17–25