bjh research paper

Oxindole alkaloids from *Uncaria tomentosa* induce apoptosis in proliferating, G0/G1-arrested and bcl-2-expressing acute lymphoblastic leukaemia cells

Nicole Bacher,[1,2,3] Martin Tiefenthaler,[4] Sonja Sturm,[5] Hermann Stuppner,[5] Michael J. Ausserlechner,[6,7] Reinhard Kofler[2,6] and Günther Konwalinka[1]

[1]*Department of Internal Medicine, Medical University of Innsbruck,* [2]*Tyrolean Cancer Research Institute,* [3]*Cell Metabolism and Differentiation Research Group, Institute for Biomedical Aging Research of the Austrian Academy of Sciences,* [4]*Department of Nephrology, Medical University of Innsbruck,* [5]*Department of Pharmacy, Faculty of Chemistry and Pharmacy, University of Innsbruck,* [6]*Division of Molecular Pathophysiology, Biocenter, Medical University of Innsbruck, and* [7]*Paediatric Department, Molecular Biology Research Laboratory, Medical University of Innsbruck, Innsbruck, Austria*

Received 22 July 2005; accepted for publication 9 November 2005

Correspondence: Prof. G. Konwalinka, MD, Department of Internal Medicine, Medical University of Innsbruck, Anichstr. 35, A-6020 Innsbruck, Austria.
E-mail: guenther.konwalinka@uibk.ac.at

Summary

Natural products are still an untapped source of promising lead compounds for the generation of antineoplastic drugs. Here, we investigated for the first time the antiproliferative and apoptotic effects of highly purified oxindole alkaloids, namely isopteropodine (A1), pteropodine (A2), isomitraphylline (A3), uncarine F (A4) and mitraphylline (A5) obtained from *Uncaria tomentosa*, a South American Rubiaceae, on human lymphoblastic leukaemia T cells (CCRF-CEM-C7H2). Four of the five tested alkaloids inhibited proliferation of acute lymphoblastic leukaemia cells. Furthermore, the antiproliferative effect of the most potent alkaloids pteropodine (A2) and uncarine F (A4) correlated with induction of apoptosis. After 48 h, 100 μmol/l A2 or A4 increased apoptotic cells by 57%. CEM-C7H2 sublines with tetracycline-regulated expression of bcl-2, p16ink4A or constitutively expressing the cowpox virus protein crm-A were used for further studies of the apoptosis-inducing properties of these alkaloids. Neither overexpression of bcl-2 or crm-A nor cell-cycle arrest in G0/G1 phase by tetracycline-regulated expression of p16INK4A could prevent alkaloid-induced apoptosis. Our results show the strong apoptotic effects of pteropodine and uncarine F on acute leukaemic lymphoblasts and recommend the alkaloids for further studies in xenograft models.

Keywords: *Uncaria tomentosa*, alkaloids, apoptosis, lymphoblastic leukaemia, cell-cycle arrest.

Empirically screened agents that inhibit tumour cell growth and trigger apoptosis *in vivo* and *in vitro* are responsible for increasing survival rates amongst cancer patients. However, mutations or alterations in critical pathways, such as those controlling cell-cycle checkpoints, make tumour cells resistant to these agents. For this reason, the development of new antitumour agents, acting via distinct apoptosis pathways independent of cell-cycle phases, is a main focus of research. Natural products still are an important untapped source of promising lead compounds with unique sites of action as antineoplastic drugs. Preparations from *Uncaria tomentosa*, a South American Rubiaceae, have been used in the Peruvian traditional medicine for treatment of inflammation, various infections and cancer. Glycosides, steroids and alkaloids have been identified as constituents of the plant (Stuppner *et al*, 1992a). Besides immunomodulating and immunostimulating actions (Lemaire *et al*, 1999; Sandoval *et al*, 2000; Aguilar *et al*, 2002), antiproliferative and apoptotic effects of aqueous extracts, primarily containing carboxyl alkyl esters from *U. tomentosa*, on human myeloid leukaemia cells, such as K562 and HL60, have been described (Sheng *et al*, 1998). In addition, constituents of the plant have also been shown to exert cytotoxic activity on breast cancer cells (Riva *et al*, 2001).

The goal of this study was to investigate whether highly purified oxindole alkaloids, namely isopteropodine (A1), pteropodine (A2), isomitraphylline (A3) and uncarine F (A4) and mitraphylline (A5), obtained from *U. tomentosa* have antiproliferative and apoptotic effects on human acute lym-

© 2005 Blackwell Publishing Ltd, doi:10.1111/j.1365-2141.2005.05907.x

phoblastic leukaemia (ALL) (CEM-C7H2) T cells. The apoptotic effects of the most potent alkaloids were quantified and the underlying pathways investigated. Two distinct signal transduction pathways leading to apoptosis are well known: the intrinsic mitochondrial pathway, which is activated by a variety of cellular signals and mainly regulated by members of the bcl-2 family, and the extrinsic, receptor-mediated pathway with its ligand–receptor interaction recruiting transducer proteins to activate the apoptosis machinery (Strasser *et al*, 2000).

In the intrinsic mitochondrial pathway, upregulation of bcl-2 prevents caspase activation and apoptosis in several, though not all systems (Coultas & Strasser, 2003). To study the effects of bcl-2 on alkaloid-induced apoptosis in CEM leukaemia cells, a stably transfected subclone was used that allowed tetracycline-regulated expression of bcl-2. In this system, glucocorticoid-induced cell death could be delayed by 24 h by expression of bcl-2 (Hartmann *et al*, 1999). Friesen *et al* (1996) have suggested that certain chemotherapeutic agents, such as doxorubicin, might induce apoptosis via the CD95/Fas receptor pathway. To find out whether the purified alkaloids induce programmed cell death via the CD95/Fas pathway, crm-A overexpressing CEM cells were used. The cowpox virus protein crm-A has previously been shown to specifically inhibit caspase-8 and thereby almost completely prevents CD95/Fas-induced poly (ADP-ribose) polymerase (PARP) cleavage and apoptosis (Geley *et al*, 1997). Drug resistance of cancer cells to chemotherapeutic agents can be partly explained by cell-cycle arrest in the G1-phase. Almost all conventional anticancer drugs are primarily effective against rapidly dividing cells (Banker *et al*, 1998). Compared with proliferating lymphocytes, a 500-fold higher concentration of cytosine arabinoside, 6-thioguanine, 5-fluorouracil and hydroxyurea is required to kill resting cells (Carson *et al*, 1983). In order to test whether the purified alkaloids from *U. tomentosa* also induce apoptosis in G0/G1-arrested leukaemia cells, a cyclin-dependent kinase (CDK) inhibitor p16INK4A-expressing CEM cell line was used. After doxycycline treatment, this subline showed stable arrest in the G0/G1 phase of cell cycle (Ausserlechner *et al*, 2001).

We report here that pteropodine (A2) and uncarine F (A4) inhibit proliferation of ALL cells by induction of apoptosis. Neither overexpression of bcl-2 nor crm-A nor cell-cycle arrest in G0/G1 phase of cell cycle could prevent alkaloid-induced apoptosis.

Materials and methods

Chemicals

The alkaloids isopteropodine (A1), pteropodine (A2), isomitraphylline (A3), uncarine F (A4) and mitraphylline (A5) were isolated by column chromatography and identified by spectroscopic methods (Sturm, 1993). The compounds were dissolved in dimethyl sulphoxide (DMSO) and stored at −20°C, the purity of the alkaloids (>99%) was verified by high performance liquid chromatography (HPLC) (Stuppner *et al*, 1992b). As the amounts of alkaloids A2 and A4 were limited, not all experiments could be carried out with both. Doxycycline-hydrochloride and dexamethasone (Dex)-cristalline were obtained from Sigma, Vienna, Austria. The caspase inhibitors caspase-8 inhibitor (C8-INH) Z-IETD-FMK and caspase-9 inhibitor (C9-INH) Z-LEHD-FMK were purchased from R&D Systems, McKinley, MN, USA).

Cell lines

CCRF-CEM-C7H2, CEM-C7H2-10E1 (with doxycycline-regulated expression of bcl-2) (Strasser-Wozak *et al*, 1995), CEM-C7H2-6E2 (with doxycycline-regulated p16INK4A expression) (Ausserlechner *et al*, 2001), CEM-C7H2-2E8 (constitutively expressing the cowpox virus protein crm-A) and its control line CEM-C7H2-5B3 (Geley *et al*, 1997) have been published. In CEM-C7H2-10E1, transgenic bcl-2 expression is abrogated in the presence of 100 ng/ml doxycycline (Hartmann *et al*, 1999). In CEM-C7H2-6E2, G0/G1 arrest was induced by addition of 200 ng/ml doxycycline for 24 h as formerly described (Ausserlechner *et al*, 2001). All cell lines were mycoplasma-free and their authenticity was verified by DNA fingerprinting (Parson *et al*, 2005). All cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium containing 5 or 10% heat-inactivated fetal calf serum, 1% L-glutamine, 100 U/ml penicillin 100 μg/ml streptomycin and 250 ng/ml fungizone at 5% CO_2 and 37°C in saturated humidity. Cells from exponentially grown culture were used for all experiments.

^{3}H-thymidine proliferation assay

Cells were cultured at a density of 10 000/well in triplicate in 96-well plates in 200 μl medium or drugs, made up in medium at different concentrations. The plates were incubated at 5% CO_2 and 37°C in saturated humidity. After 48 h, cells were pulsed with ^{3}H-thymidine at 74 kBq/well and incubated for a further 20 h. The cells were collected on glass fibre paper with an automatic harvester (Mash II, Flow Laboratories, Bonn, Germany) and the ^{3}H-thymidine uptake was measured using a Beckman liquid scintillation counter.

Methyl thiazol tetrazolium (MTT)-proliferation assay

The MTT assay is based on the cleavage of yellow tetrazolium salt, which turns to purple formozan crystals by viable, metabolic active cells (Mosmann, 1983). Cells were seeded at a density of 10 000/well in triplicates in 96-well plates in a final volume of 100 μl medium containing different concentrations of drugs. Cell culture medium without cells was used as a blank. After 48 h, 10 μl thiazolyl blue tetrazolium bromide (Sigma) [5 mg/ml in phosphate-buffered saline (PBS)] were added to each well and the plates were incubated for another 4 h in the cell culture incubator at 37°C. For solubilisation, 100 μl 10% sodium dodecyl sulphate (SDS)/0·01 N HCl were added to each well and the plates were incubated overnight at

 © 2005 Blackwell Publishing Ltd,

room temperature in the dark. Absorbance was measured in a microplate reader (Biorad, Hercules, CA, USA) at 570 nm with a reference wavelength at 655 nm.

Measurement of apoptosis

Annexin-V/propidium iodide (PI) assay Cells were washed in PBS and stained with annexin-V-fluorescein isothiocyanate (FITC)/PI, using the Apoptosis Detection Kit (Alexis, Grünberg, Germany) according to the manufacturers' instructions: approximately, 1×10^5 cells were washed in PBS, resuspended in 200 µl annexin-V-binding buffer, containing 5 µl annexin-V-FITC, and incubated for 10 min in the dark. Cells were washed and then resuspended in 100 µl annexin-V-binding buffer. Another 100 µl annexin-V-binding buffer containing 2 mg/ml PI were added before fluorescence was determined by flow cytometry.

PI staining

Cells were centrifuged and the pellets were resuspended with 200 µl PI solution (50 mg/ml PI, 0·1% sodium citrate, 0·1% Triton-X 100). Cellular DNA content was measured by flow cytometry. The nuclei in the sub-G1 marker window were considered to represent apoptotic cells (Nicoletti *et al*, 1991).

Western blot analysis of PARP and caspase-9

After treatment, total protein was extracted from $2{\cdot}5 \times 10^6$ cells with lysis buffer (50 mmol/l Tris, pH 8, 1% nonidet P40, 0·5% deoxycholate, 150 mmol/l sodium chloride, 10 µg/ml leupeptin, 100 µmol/l phenylmethylsulphonyl fluoride (PMSF), 1·46 mmol/l pepstatin A). After 30 min on ice, insoluble material was eliminated by centrifugation. Equal amounts of protein were separated by 12·5% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and electrophoretically transferred onto a nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden). Protein-binding sites were saturated with 5% powdered non-fat milk in Tris-buffered saline (TBS) supplemented with Tween 0·1% (TBS-T) for 1 h. The membranes were incubated overnight with polyclonal rabbit antibodies against human PARP (Promega, Madison, WI, USA) and caspase-9 (New England Biolabs, Ipswich, MA, USA) or monoclonal mouse β-actin antibody (Sigma) in TBS-T followed by a 1-h exposure to horseradish peroxidase-conjugated antimouse antibody (Promega) or rabbit IgG (Boehringer, Mannheim, Germany) in TBS-T. Peroxidase activity was detected using the chemoluminescence detection kit Western blotting luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Statistical analysis

For all experimental data, mean and SD were calculated, and significant differences were determined using an unpaired Student's *t*-test. *P*-values <0·05 were considered significant.

Results

Effects of alkaloids A1 to A5 on proliferation of CEM-C7H2 cells

As aqueous extracts of *U. tomentosa* have already been shown to exert inhibitory effects on the proliferation of human tumour cells, we examined the effects of five purified alkaloids (A1–A5) on ALL T cells. In our first experiments, the effects of increasing concentrations of A1–A5, ranging from 12·5 to 100 µmol/l on CEM-C7H2 cells were measured by ^{3}H-thymidine uptake assay. A sigmoid dose–response curve was found for A1, A2, A3 and A4 but not for A5 (Fig 1). The alkaloids A2 and A4 exhibited the most potent cytostatic effect on leukaemia cells. After 48 h, these two alkaloids inhibited the proliferation of CEM-C7H2 cells by more than 95% at a concentration of 100 µmol/l. Therefore, only A2 and A4 were used for further experiments.

A2 and A4 induce apoptosis in CEM-C7H2 cells

To investigate whether the A2- and A4-induced inhibition of cell proliferation correlated with apoptotic cell death, PI staining was performed. For this purpose, CEM-C7H2 cells were incubated for 24, 48 and 72 h with increasing concentrations (50–200 µmol/l) of A2 and A4. Dex, a standard chemotherapeutic agent for T-ALL cells, served as positive control. As shown in Fig 2A, a dose of 50 µmol/l A2 or A4 increased the sub-G1 population after 24 h by 31% and 13% respectively compared with controls. At this concentration, an extension of the incubation period to 48 or 72 h did not lead to a significant increase in apoptosis. However, a significant dose- and time-dependent increase in apoptosis could be observed when CEM-C7H2 cells were treated with 100 and 200 µmol/l A2 or A4. Thus, with 100 µmol/l A2, the apoptosis rates increased from 47% after 24 h to 68% and 81% after 48 and 72 h, respectively. With 200 µmol/l A2, the apoptotic rates at various time points further increased. Similar results could be observed with A4. DMSO in a concentration used for dissolving the alkaloids (0·73%) had no apoptotic effect.

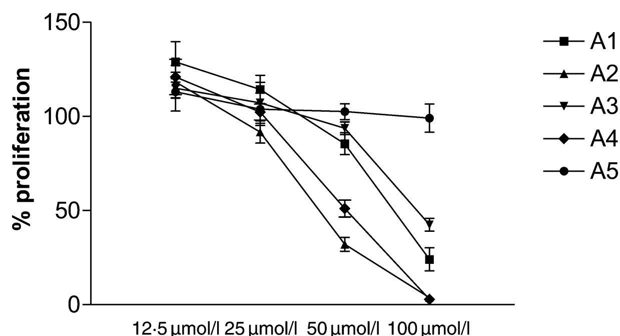

Fig 1. Effect of the alkaloids A1–A5 on proliferation of CEM-C7H2 cells. Cells were treated with indicated concentrations of A1, A2, A3, A4 and A5 for 48 h. Percentage proliferation was measured by ^{3}H-thymidine proliferation assay.

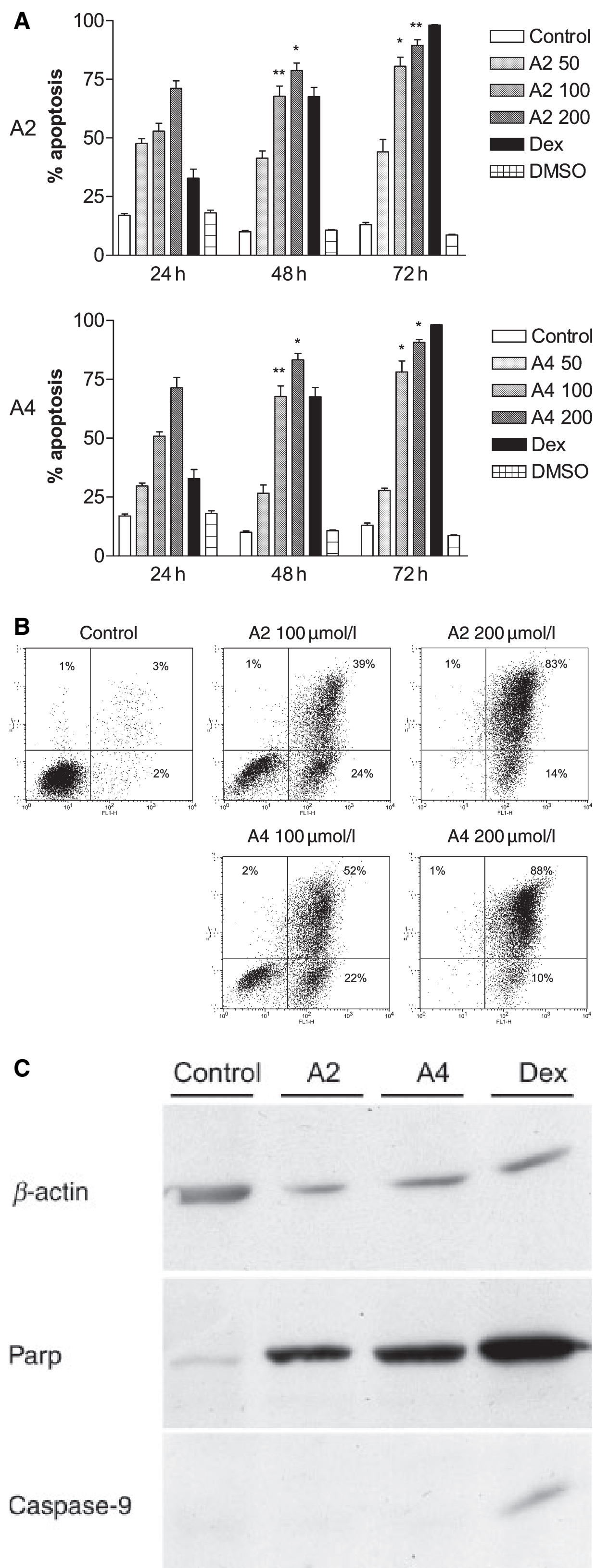

Fig 2. Induction of apoptosis in CEM-C7H2 cells by A2 and A4. (A) For PI-staining cells were incubated with 50, 100 and 200 µmol/l A2 or A4 for 24, 48 and 72 h. Dex (100 nmol/l) and DMSO at a concentration used for dissolving the alkaloids (0·73%) served as controls. After harvesting, cells were stained with PI and subjected to FACS analysis. Each panel represents the mean (±SD) of at least three-independent experiments. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$ for each alkaloid *versus* the previous time point. (B) For detection of annexin-V binding, CEM-C7H2 cells were treated with 100 or 200 µmol/l A2 or A4 for 48 h. Cell death was measured by annexin-V-binding assay and FACS analysis. On the *x*-axis fluorescence-1 (FL-1), detecting FITC-labelled annexin-V, *versus* on the *y*-axis fluorescence-2 (FL-2), detecting PI as measure for membrane integrity, is shown. The indicated numbers show percentage of total cells. (C) Cells were treated for 48 h with 200 µmol/l A2 or A4 or 100 nmol/l Dex as control. Total protein of untreated and treated cells was prepared and analysed by Western blotting for cleaved caspase-9 (37 kDa), cleaved PARP (85 kDa) and β-actin (43 kDa) as a loading control.

In order to confirm the potent apoptotic efficiency of A2 and A4, annexin-V-binding assays have also been performed. As shown in Fig 2B, after 48 h, a concentration of 100 µmol/l increased the rates of annexin-V+ cells from 5% in control cells to 63% and 74% in A2- and A4-treated cells. At a concentration of 200 µmol/l, more than 97% of CEM-C7H2 cells showed annexin-V staining with both alkaloids.

Activation of caspase-3, a key enzyme in apoptosis, was assessed by the cleavage of one of its substrates, PARP, which also contributes to apoptosis (Simbulan-Rosenthal *et al*, 1998). As revealed by Western blotting, CEM-C7H2 cells incubated with 200 µmol/l of A2 or A4 or 100 nmol/l Dex underwent complete PARP cleavage within 48 h of exposure to alkaloid and Dex (Fig 2C).

A4-induced apoptosis in CEM-C7H2 cells is independent of bcl-2 overexpression

In the intrinsic mitochondrial pathway, apoptosis is triggered by pro-apoptotic members of the bcl-2 family, leading predominantly to activation of the initiator caspase-9. Cell death is regulated via interaction between pro-survival and -death members of the bcl-2 family, bcl-2 and its functional homologues being essential for cell survival (Coultas & Strasser, 2003). In order to test whether A4-triggered apoptotic cell death can be blocked by overexpression of bcl-2, CEM-C7H2-10E1 cells with transgenic bcl-2 expression were incubated with 100 µmol/l A4 for 48 h and the extent of apoptosis measured by PI staining with subsequent fluorescence-activated cell sorting (FACS) analysis. Results were compared with those found in CEM-C7H2-10E1 cells under repressive conditions for transgenic bcl-2 expression (i.e. in the presence of 100 ng/ml doxycycline). As Fig 3A shows, the percentage of apoptosis induced by A4 was not significantly different between bcl-2-expressing and -repressing CEM-C7H2-10E1 cells. In agreement with previously published results, the percentage of apoptotic cells was significantly lower in bcl-2-overexpressing cells compared with control cells when Dex was used as the apoptosis inducer (Hartmann *et al*, 1999). As overexpression of bcl-2 could not prevent A4-induced apoptosis, it was not likely that caspase-9 was activated in

 © 2005 Blackwell Publishing Ltd,

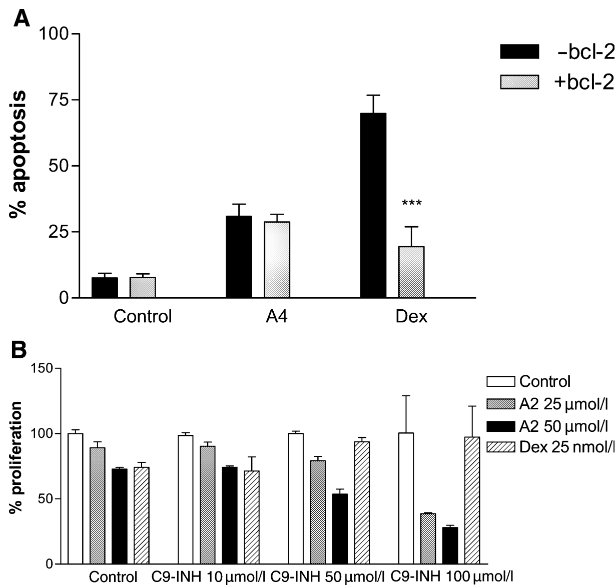

Fig 3. Overexpression of bcl-2 does not inhibit A4-induced apoptosis. (A) CEM-C7H2-10E1 cells with transgenic bcl-2 expression, which is abrogated in the presence of doxycycline, were incubated with 100 µmol/l A4 or Dex (100 nmol/l) in the presence or absence of 100 nmol/l doxycycline. Apoptosis was measured by PI staining and accompanied FACS analysis. Data represent the mean (±SD) of at least three-independent experiments. $^{***}P < 0{\cdot}001$ for Dex in bcl-2-expressing *versus* -repressing cells. (B) CEM-C7H2 cells were incubated with indicated concentrations of A2, dexamethasone (Dex) and the caspase-9 inhibitor (C9-INH) in the indicated concentrations. Percentage proliferation was measured by an MTT-assay. Data represent the mean (±SD) of triplicate determinations.

alkaloid-treated cells. In order to test this hypothesis, cleaved caspase-9 was measured in CEM-C7H2 cells after a 48 h exposure to A2, A4 or Dex. As can be seen in Fig 2C, activated caspase-9 was only observed in Dex-treated cells, whereas no cleaved products of caspase-9 could be detected after A2 and A4 treatment. To further confirm the independence of alkaloid-induced apoptosis from the intrinsic mitochondrial pathway, C9-INH studies have been performed. CEM-C7H2 cells were incubated with 25 and 50 µmol/l A2 or, as a control, Dex in the absence or presence of increasing concentrations of the C9-INH Z-LEHD-FMK. After 48 h, proliferation was measured by the MTT assay. As shown in Fig 3B, without C9-INH, the proliferation of CEM-C7H2 cells was inhibited by 25 µmol/l and 50 µmol/l A2 as well as Dex. Increasing concentrations of C9-INH completely abolished Dex-induced inhibition of proliferation but did not diminish the A2-induced inhibition of proliferation.

Apoptosis induced by A2 is independent of CD95/Fas signalling

As it has been suggested that chemotherapy-induced apoptosis can occur through the CD95/Fas pathway (Friesen *et al*, 1996), we investigated whether this proteolytic pathway was operative in A2-induced apoptosis. It is known that the cowpox virus protein crm-A inhibits in particular caspase-8 in particular, and thereby almost completely blocks CD95/Fas-induced apoptosis. To determine whether A2 acts via this pathway, we used crm-A-expressing C7H2-2E8 and crm-A negative control cells (C7H2-5B3). Cells incubated with activating anti-Fas antibodies (Beckman Coulter, Fullerton, CA, USA) for 26 h revealed apoptosis only in crm-A negative cells but not in crm-A expressing cells, showing that crm-A expression was sufficient to repress CD95/Fas-induced apoptosis (Fig 4A). In contrast, with 200 µmol/l A2 for 48 h, comparable rates of apoptosis were found in crm-A expressing and in control cells, suggesting that A2 does not act via the CD95/Fas pathway. To further test this hypothesis, C8-INH studies have been performed. To this end, CEM-C7H2 cells were incubated with 25 and 50 µmol/l A2 or, as a control, activating anti-Fas antibodies in absence or presence of increasing concentrations of the C8-INH Z-IETD-FMK and proliferation was measured by MTT assay after 48 h. The results presented in Fig 4B clearly illustrate that C8-INH diminished the strong antiproliferative effect of anti-Fas antibodies in a concentration-dependent manner. Thus, when cells were exposed to anti-Fas antibodies, proliferation was inhibited by 100%; C8-INH in concentrations of 10 µmol/l, 50 µmol/l and 100 µmol/l could attenuate this drastic effect by 8%, 60% and 78% respectively but had no effect on A2-induced inhibition of proliferation.

p16INK4A-induced G0/G1 arrest does not prevent A2 and A4-induced apoptosis

Most cytostatic agents kill dividing but not resting malignant cells. In cancer treatment, growth arrest can provide an opportunity for tumour cells to escape cell death and later on regain proliferative capacity and develop resistance to subsequent clinical interventions. To determine whether A2 and A4 can induce apoptosis in G0/G1-arrested cells, CEM-C7H2-6E2 cells were used. In these cells, doxycycline-induced expression of transgenic p16INK4A induces stable arrest in G0/G1 phase. Proliferating and arrested cells (±200 ng/ml doxycycline for 24 h) were treated with A2 and A4 in concentrations up to 200 µmol/l for 48 h prior to PI staining. Dex was used as positive control. A low concentration (100 µmol/l) of both alkaloids induced programmed cell death in cycling cells but not in G0/G1-arrested C7H2-6E2 cells (Fig 5). However, even a twofold increase of alkaloids (200 µmol/l) led to a significant increase in apoptosis in G0/G1-arrested cells, comparable with that obtained in dividing cells at a concentration of 100 µmol/l.

Discussion

Here, we report for the first time on the cytostatic effects of some of the highly purified alkaloids of *U. tomentosa* on acute lymphoblastic T cells. With the exception of mitraphylline (A5), all tested alkaloids inhibited the growth of CEM-C7H2 cells in a dose-dependent manner. Pteropodine (A2) and uncarine F (A4), which induced about 50% and 100%

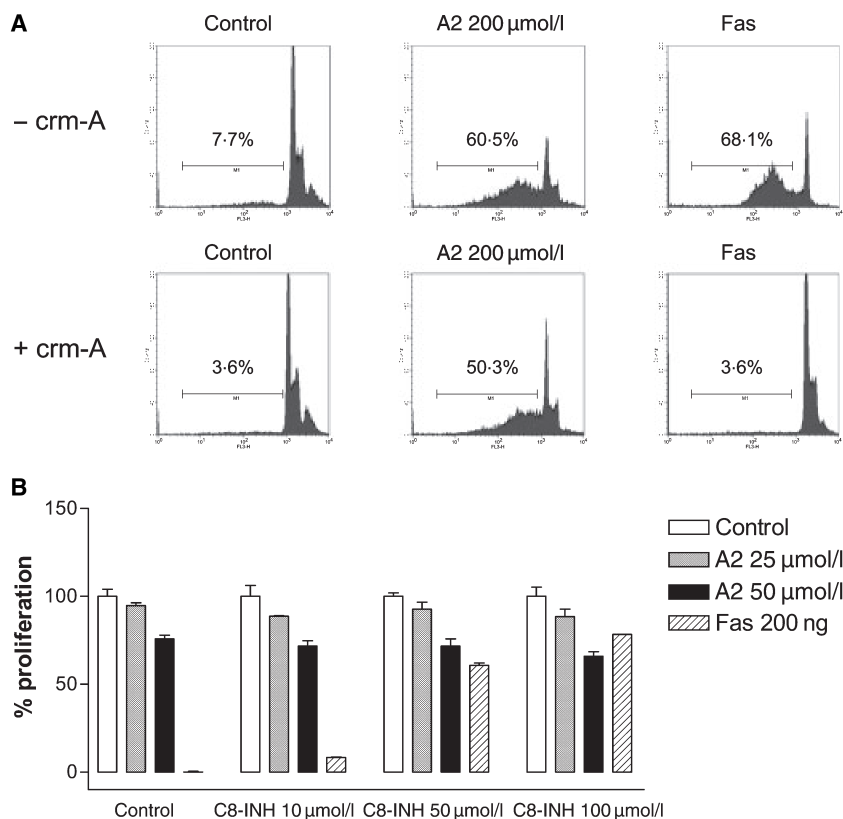

Fig 4. A2-induced apoptosis is independent of CD95/CD95-L pathway. (A) Crm-A-expressing CEM-C7H2-2E8 cells and control cells (C7H2-5B3) were incubated with 200 µmol/l A2 for 48 h or with a monoclonal antibody to CD95/Fas (Fas) for 26 h, stained with PI and analysed by FACS. The indicated numbers show percentage of apoptosis of total cells. (B) CEM-C7H2 cells were incubated with A2, monoclonal CD95/Fas antibody (Fas) and the caspase-8 inhibitor (C8-INH) in the indicated concentrations. Percentage proliferation was measured by an MTT-assay. Data represent the mean (±SD) of triplicate determinations.

inhibition of CEM cells at 50 µmol/l and 100 µmol/l respectively, exerted the strongest cytostatic effect (Fig 1).

Chemotherapeutic drugs are considered to kill tumour cells by activating a cascade of events resulting in programmed cell death. In agreement with this line of thought, we provide evidence that the purified alkaloids induced a cell death with the classic features of apoptosis. Thus, cell death was associated with DNA fragmentation, as detected by PI incorporation in nuclei, and externalisation of the outer leaflet of the membrane, as shown by annexin-V staining (Fig 2A,B). In addition, PARP cleavage, a typical indicator of caspase activation, was shown in CEM-C7H2 cells after A2 and A4 treatment (Fig 2C).

To further characterise the apoptosis pathway activated by alkaloid treatment, a series of experiments was performed with the most potent alkaloids, namely pteropodine (A2) and uncarine F (A4).

In the intrinsic mitochondrial pathway, apoptosis is triggered by pro-apoptotic members of the bcl-2 family, leading predominantly to activation of the initiator caspase-9. This pathway plays a major role in drug-induced apoptosis and bcl-2 overexpression not only inhibits anticancer drug-induced apoptosis in short-term assays, but also promotes long-term survival and continued clonogenic growth (Strasser *et al*, 1994; Schmitt *et al*, 2000). Overexpression of antiapoptotic bcl-2 probably occurs in more than half of all haematological malignancies rendering neoplastic cells resistant to most cytotoxic anticancer drugs (Carroll *et al*, 2003). In our experiments, overexpression of bcl-2 could not prevent alkaloid-induced apoptosis in CEM cells (Fig 3A), although it delayed cell death induced by glucocorticoids as previously described (Hartmann *et al*, 1999). In agreement with these results, the caspase-9 inhibitor Z-LEHD-FMK could not reduce the alkaloid-induced inhibition of proliferation but completely abolished inhibition of proliferation in Dex-treated cells (Fig 3B). Moreover, activation of caspase-9 as a large active 37 kDa subunit was only observed in Dex-treated control cells but not after alkaloid-treatment (Fig 2C). The inability of bcl-2 to block A4-induced apoptosis might not be surprising as it does not interfere with all apoptotic stimuli (Reed, 1994). Considerable evidence has suggested that cytotoxic drugs with different mechanisms of action, such as cisplatin, bleomycin and methotrexate, exert their effects via the CD95/Fas receptor ligand system (Muller *et al*, 1997). CD95/Fas-induced apoptosis can be blocked specifically by the cowpox virus serpin crm-A, which potently inhibits caspase-8, the major initiator caspase of the extrinsic pathway (Strasser

 © 2005 Blackwell Publishing Ltd,

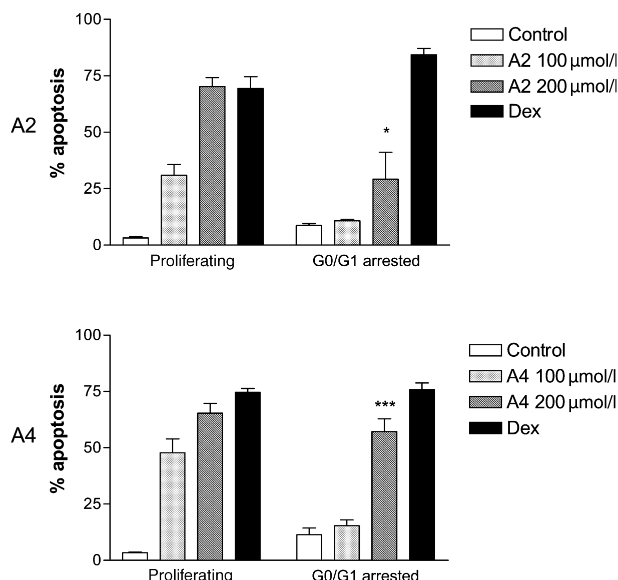

Fig 5. G0/G1 arrest does not prevent A2/A4-induced apoptosis. For cell-cycle arrest, CEM-C7H2-6E2 cells were incubated with or without 200 ng/ml doxycycline for 24 h and thereafter treated with A2 or A4 for another 48 h. Dexamethasone was used as a positive control. After harvesting, cells were stained with PI and subjected to FACS analysis. Each panel represents the mean (±SD) of at least three-independent experiments. $^{*}P < 0{\cdot}05$; $^{***}P < 0{\cdot}001$ for alkaloid-treated *versus* control cells in G0/G1 arrest.

et al, 2000). The degree of apoptosis induced by the alkaloids was not reduced in the crm-A-expressing cell line, suggesting that the extrinsic pathway may not be essential for pteropodine-induced apoptosis (Fig 4A). Furthermore, C8-INH studies revealed that alkaloid-induced inhibition of proliferation could not be abrogated by the C8-INH (Fig 4B). Interestingly, bcl-2 as well as crm-A overexpression failed to affect alkaloid-induced apoptosis and neither caspase-9 nor C8-INH abolished the alkaloids effects. Thus, the exact mechanism for the induction of apoptosis by A2 and A4 remains unclear.

Most chemotherapeutic agents selectively induce apoptosis in replicating cells. But if cell-cycle arrest is induced in cancer cells, they may escape the effects of cytostatic drugs and continue their proliferative activity after the withdrawal of the drug. Thus, for instance, the chemotherapeutic agents paclitaxel and vincristine failed to induce apoptosis in human ovarian cancer cells when arrested in the G0/G1 phase of cell cycle by p16INK4A transduction (Kawakami *et al*, 2001). In contrast, the alkaloids A2 and A4 tested in our study induced apoptosis in proliferating as well as G0/G1-arrested cells. Furthermore, the concentration of alkaloids required for killing arrested cells was just twice that for proliferating cells (Fig 5) in contrast to cytosine arabinoside, 6-thioguanine, 5-fluorouracil and hydroxyurea, which required 500-fold higher concentrations to kill resting lymphocytes (Banker *et al*, 1998). In conclusion, pteropodine (A2) and uncarine F (A4) exerted cytotoxic effects on acute leukaemic lymphoblastic cells by induction of apoptosis in both, proliferating and G0/G1-arrested stages. In addition, bcl-2 overexpression did not prevent the cells from programmed cell death. Based on these characteristics in *in vitro* systems, investigation of the alkaloids in xenograft models appears to be warranted.

Acknowledgements

We thank Hanni Linert and Michaela Karches-Böhm for their excellent technical assistance. This work was supported by grants from the Austrian Science Funds (SFB-F021 and FWF-P15383) and the Austrian Cancer Society – Tyrol to Werner Zwerschke. The Tyrolean Cancer Research Institute and this study were supported by donations from the 'Tiroler Landeskrankenanstalten Ges.m.b.H. (TILAK), the Tyrolean Cancer Society', various businesses, financial institutions and the people of Tyrol.

References

Aguilar, J.L., Rojas, P., Marcelo, A., Plaza, A., Bauer, R., Reininger, E., Klaas, C.A. & Merfort, I. (2002) Anti-inflammatory activity of two different extracts of *Uncaria tomentosa* (Rubiaceae). *Journal of Ethnopharmcology*, **81,** 271–276.

Ausserlechner, M.J., Obexer, P., Wiegers, G.J., Hartmann, B.L., Geley, S. & Kofler, R. (2001) The cell cycle inhibitor p16(INK4A) sensitises lymphoblastic leukaemia cells to apoptosis by physiologic glucocorticoid levels. *Journal of Biological Chemistry*, **276,** 10984–10989.

Banker, D.E., Groudine, M., Willman, C.L., Norwood, T. & Appelbaum, F.R. (1998) Cell cycle perturbations in acute myeloid leukaemia samples following in vitro exposures to therapeutic agents. *Leukaemia Research*, **22,** 221–239.

Carroll, W.L., Bhojwani, D., Min, D.J., Raetz, E., Relling, M., Davies, S., Downing, J.R., Willman, C.L. & Reed, J.C. (2003) Paediatric acute lymphoblastic leukaemia. *Hematology*, American Society of Hematology Education Program, 102–131.

Carson, D.A., Wasson, D.B., Teatle, R. & Yu, A. (1983) Specific toxicity of 2-chlorodeoxyadenosine toward resting and proliferating human lymphocytes. *Blood*, **62,** 737–743.

Coultas, L. & Strasser, A. (2003) The role of the bcl-2 protein family in cancer. *Seminars in Cancer Biology*, **13,** 115–123.

Friesen, C., Herr, I., Krammer, P.H. & Debatin, K.M. (1996) Involvement of the CD95 (Apo-1/Fas) receptor/ligand system in drug-induced apoptosis in leukaemia cells. *Nature Medicine*, **2,** 574–577.

Geley, S., Hartmann, B.L., Kapelari, K., Egle, A., Villunger, A., Heidacher, D., Greil, R., Auer, B. & Kofler, R. (1997) The interleukin 1-converting enzyme CrmA prevents Apo1/Fas- but not glucocorticoid-induced poly(ADP-ribose) polymerase cleavage and apoptosis in lymphoblastic leukaemia cells. *FEBS Letters*, **402,** 36–40.

Hartmann, G.L., Geley, S., Loffler, M., Hattmannstorfer, R., Strasser-Wozak, E.M., Auer, B. & Kofler, R. (1999) Bcl-2 interferes with the execution phase, but not upstream events, in glucocorticoid-induced leukaemia apoptosis. *Oncogene*, **18,** 713–719.

Kawakami, Y., Hama, S., Hiura, M., Nogawa, T., Chiba, T., Yokoyama, T., Takashima, S., Tajiri, H., Eguchi, K., Nagai, N., Shigemasa, K., Ohama, K., Kurisu, K. & Heike, Y. (2001) Adenovirus-mediated p16 gene transfer changes the sensitivity to taxanes and Vinca alkaloids of human ovarian cancer cells. *Anticancer Research*, **21,** 2537–2545.

Lemaire, I., Assinewe, V., Cano, P., Awang, D.V. & Arnason, J.T. (1999) Stimulation of interleukin-1 and -6 production in alveolar macrophages by the neotropical liana, *Uncaria tomentosa* (una de gato). *Journal of Ethnopharmacology*, **64,** 109–115.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, **65,** 55–63.

Muller, M., Strand, S., Hug, H., Heinemann, E.M., Walczak, H., Hofmann, W.J., Stremmel, W., Krammer, P.H. & Galle, P.R. (1997) Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/ligand system and involves activation of wild-type p53. *Journal of Clinical Investigation*, **99,** 403–413.

Nicoletti, I., Migliorati, G., Pagliacci, M.C., Grignani, F. & Riccardi, C. (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *Journal of Immunological Methods*, **139,** 271–279.

Parson, W., Kirchebner, R., Muhlmann, R., Renner, K., Kofler, A., Schmidt, S. & Kofler, R. (2005) Cancer cell line identification by short tandem repeat profiling: power and limitations. *FASEB Journal*, **19,** 434–436.

Reed, J.C. (1994) Bcl-2 and the regulation of programmed cell death. *Journal of Cellular Biology*, **124,** 1–6.

Riva, L., Cordini, D., Di Fronzo, G., De Feo, V., De Tommasi, N., De Simone, F. & Pizza, C. (2001) The antiproliferative effects of *Uncaria tomentosa* extracts and fractions on the growth of breast cancer cell line. *Anticancer Research*, **21,** 2457–2461.

Sandoval, M., Charbonnet, R.M., Okuhama, N.N., Roberts, J., Krenova, Z., Trentacosti, A.M. & Miller, M.J. (2000) Cat's claw inhibits TNFalpha production and scavenges free radicals: role in cytoprotection. *Free Radical Biology*, **29,** 71–78.

Schmitt, C.A., Rosenthal, C.T. & Lowe, S.W. (2000) Genetic analysis of chemoresistance in primary murine lymphomas. *Nature Medicine*, **6,** 1029–1035.

Sheng, Y., Pero, R.W., Amiri, A. & Bryngelsson, C. (1998) Induction of apoptosis and inhibition of proliferation in human tumour cells treated with extracts of *Uncaria tomentosa*. *Anticancer Research*, **18,** 3363–3368.

Simbulan-Rosenthal, C.M., Rosenthal, D.S., Iyer, S., Boulares, A.H. & Smulson, M.E. (1998) Transient poly(ADP-ribosyl)ation of nuclear proteins and role of poly(ADP-ribose) polymerase in the early stages of apoptosis. *Journal of Biological Chemistry*, **273,** 13703–13712.

Strasser, A., Harris, A.W., Jacks, T. & Cory, S. (1994) DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by Bcl-2. *Cell*, **79,** 329–339.

Strasser, A., O'Connor, L. & Dixit, V.M. (2000) Apoptosis signalling. *Annual Review of Biochemistry*, **69,** 217–245.

Strasser-Wozak, E.M., Hattmannstorfer, R., Hala, M., Hartmann, B.L., Fiegl, M., Geley, S. & Kofler, R. (1995) Splice site mutation in the glucocorticoid receptor gene causes resistance to glucocorticoid-induced apoptosis in a human acute leukaemic cell line. *Cancer Research*, **55,** 348–353.

Stuppner, H., Sturm, S. & Konwalinka, G. (1992a) Capillary electrophoretic analysis of oxindole alkaloids from *Uncaria tomentosa*. *Journal of Chromatography*, **609,** 375.

Stuppner, H., Sturm, S. & Konwalinka, G. (1992b) HPLC analysis of the main oxindole alkaloids from *Uncaria tomentosa*. *Chromatographia*, **34,** 597–600.

Sturm, S. (1993) UV, IR, MS, one- and two-dimensional ^{1}H- and ^{13}C-NMR. Dissertation. University of Innsbruck, Innsbruck.

 © 2005 Blackwell Publishing Ltd, *British Journal of Haematology*, **132**, 615–622