Antiproliferative and Pro-apoptotic Effects of *Uncaria tomentosa* in Human Medullary Thyroid Carcinoma Cells

BEATE RINNER[1,2], ZENG XIA LI[1,3], HELGA HAAS[1], VERONIKA SIEGL[1], SONJA STURM[4], HERMANN STUPPNER[4] and ROSWITHA PFRAGNER[1]

[1]*Institute of Pathophysiology and Immunology, Center of Molecular Medicine, and*
[2]*Center for Medical Research, Core Facility Flow Cytometry, Medical University of Graz, Graz, Austria;*
[3]*Department of Biochemistry and Molecular Biology,*
Shanghai Medical School, Fudan University, Shanghai, P.R. China;
[4]*Institute of Pharmacy, Center of Molecular Biosciences, University of Innsbruck, Innsbruck, Austria*

Abstract. *Medullary thyroid carcinoma (MTC), a rare calcitonin-producing tumor, is derived from parafollicular C-cells of the thyroid and is characterized by constitutive Bcl-2 overexpression. The tumor is relatively insensitive to radiation therapy as well as conventional chemotherapy. To date, the only curative treatment is the early and complete surgical removal of all neoplastic tissue. In this study, the antiproliferative and pro-apoptotic effects of fractions obtained from Uncaria tomentosa (Willd.) DC, commonly known as uña de gato or cat's claw were investigated. Cell growth of MTC cells as well as enzymatic activity of mitochondrial dehydrogenase was markedly inhibited after treatment with different fractions of the plant. Furthermore, there was an increase in the expressions of caspase-3 and -7 and poly(ADP-ribose) polymerase (PARP) fraction, while bcl-2 overexpression remained constant. In particular, the alkaloids isopterpodine and pteropodine of U. tomentosa exhibited a significant pro-apoptotic effect on MTC cells, whereas the alkaloid-poor fraction inhibited cell proliferation but did not show any pro-apoptotic effects. These promising results indicate the growth-restraining and apoptotic potential of plant extracts against neuroendocrine tumors, which may add to existing therapies for cancer.*

Medullary thyroid carcinoma (MTC) is a calcitonin-producing tumor of the parafollicular C-cells, accounting for 5-10% of all thyroid carcinomas. The tumor is relatively insensitive to radiation therapy as well as chemotherapy. To date, the only effective treatment is the early and total surgical removal of all neoplastic tissue (1-3).

For a long time, plant-derived compounds have played an important role in drug discovery and development of agents against cancer, in particular against tumour cells, which are resistant to established drugs (4-9). One of the best known and, accordingly, also critically discussed plant extracts used in alternative cancer therapies in Europe is derived from mistletoe (10-14). However, a number of other medicinal plants, such as *Nerium oleander* L., *Millingtonia hortensis* L. and *Aegle marmelos* (Correa) L., to name just a few, were investigated for their protective effects against tumor cells (15-17). Newman *et al*. analyzed the origin of all FDA approved anticancer drugs from the 1940s until 2006 and demonstrated that plant constituents and knowledge about natural products had a great influence in the discovery and development of new anticancer drugs (18). For example, the terpene paclitaxel was isolated from *Taxus brevifolia* Nutt., the vinca alkaloids from *Catharanthus roseus*, and the DNA topoisomerase I inhibitor camptothecin from *Camptotheca acuminata* (19, 20). Recently, our group showed that plant extracts of the genera *Stemona* (Stemonaceae), *Aglaia* (Meliaceae), *Artemisia* (Asteraceae) and *Cautleya* (Zingiberaceae) were effective against MTC (4, 5, 21).

Recently, oxindole alkaloids from *Uncaria tomentosa* (Willd.) DC., a South American Rubiaceae, which has been used for treatment of rheumatoid arthritis, inflammation, infections and cancer (22-27), were found to induce apoptosis in acute lymphoblastic leukaemia cells (23). In the present study, we examined whether different extracts and fractions of *U. tomentosa* (*UNC*), induced antiproliferative and apoptitic effects in cell lines derived from different types of solid tumours, and focused our interest on MTC.

Correspondence to: Roswitha Pfragner, Department of Pathophysiology and Immunology, Center of Molecular Medicine, Medical University of Graz, Heinrichstrasse 31, A-8010 Graz, Austria. Tel: +43 3163804297, Fax: +43 3163809640, e-mail: roswitha.pfragner@medunigraz.at

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Materials and Methods

Plant materials and preparation. *UNC* fractions were kindly provided by Professor H Stuppner, Innsbruck, Austria. A crude methanolic extract (M), as well as the two isolated alkaloids isopteropodine (A1), pteropodine (A2) from *UNC* were investigated. The purity (>98%) of A1 and A2 was confirmed by high performance liquid chromatography (28). All samples were dissolved in dimethyl sulphoxide (DMSO) and stored at –20˚C (10 mg/ml).

Cell lines and culture. MTC-SK, human MTC (29, 30) cell line, was derived from a sporadic MTC of a 51-year-old central European woman. Cells were cultured in Ham's F12 medium (Biowhittaker, Verviers, Belgium) containing 10% foetal bovine serum (FBS; PAA Laboratories, Pasching, Austria). The initial cell density was 2×10^5 cells/ml. Three tumor cell lines served as controls: MCF7, human breast adenocarcinoma, pleural effusion (ATCC HTB 22), maintained in Dulbecco's modified Eagle's minimum essential medium (DMEM; Biowhittaker) without phenol red, supplemented with 5% FBS (PAA); HBMC, human melanoma Bowes (LCG Promochem/ATCC CRL-9607), maintained in Eagle's MEM (EMEM; Biowhittaker) with 10% FBS; and KNA, human adrenal pheochromocytoma cells (31) of a 73-year-old woman with MEN2A-2 syndrome, maintained in Ham's F12 with 10% FBS. HF-SAR, human skin fibroblasts isolated from a 2-year-old male (Pfragner, pers. comm.) served as normal control, and were cultured in DMEM (Biowhittaker) with 10% FBS (PAA). Initial cell density of control cell lines was 2×10^5 cells/ml. All cell lines were incubated without antibiotics at 37˚C in 5% CO_2 atmosphere. All cell lines were *Mycoplasma* free.

For experiments, cells were transferred into microplates (24 wells, flat bottom, Sarstedt, Wiener Neudorf, Austria). Tumor cells (2×10^5 cells/ml) and HF-SAR (1×10^5 cells/ml) cells were treated with different *UNC* concentrations (50-200 μM) at different times (4, 8, 12, 24 and 48h).

Cell counting. Growth kinetics and viability were investigated using the CASY-1® Cell Counter Analyser TTC (Schärfe System GmbH, Reutlingen, Germany). For cell counting, each sample was analyzed in triplicate.

WST-1 cell proliferation assay. The WST-1 assay (Roche Diagnostics GmbH, Vienna, Austria) is based on the cleavage of the tetrazolium salt WST-1 to formazan by mitochondrial dehydrogenase. The amount of formazan dye directly correlates with the number of metabolically active cells in the culture. Quantification of the formazan dye was performed by optical density measurements at 450/650 nm by an ELISA reader (Molecular Devices Corporation, Sunnyvale, CA, USA). The WST-1 assay was routinely performed together with thedetermination of cell counts. Cells were transferred into 96-well microplates with flat bottoms (Sarstedt) in a final volume of 100 μl/well, to which 10 μl/well Cell Proliferation Reagent WST-1 was added. The appropriate incubation time with the Cell Proliferation Reagent WST-1 was determined to be 120 min for the MTC-SK cell line, and 60 min for the human fibroblasts. Cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2 and shaken thoroughly for 1 min on a shaker before measurement.

LDH assay for cell viability. The CytoTox-One™ assay (Promega Corporation, Madison, WI, USA) measures the release of lactate dehydrogenase (LDH) from cells with a damaged cell membrane. MTC-SK and control cells were transferred into 96 well-microplates with flat bottoms in a final volume of 100 μl/well. Cytotox-One reagent was added to each well and incubated for 10 min. Stop solution was added and fluorescent signals were measured using 560 m exitatioan and 590 nm emission filter set. The assay results in the conversion of resazurin into a fluorescent resorufin product. The amount of the fluorescence produced is proportional to the number of lysed cells.

DAPI stain. Chromatin condensation, nuclear shrinkage and formation of apoptotic bodies can easily be observed under fluorescence microscopy, after staining of nuclei with DAPI a DNA-specific fluorochrome. A total of 2×10^5 MTC-SK cells/ml were treated with the respective plant extracts. Untreated cells served as a positive control. The cells were washed with HBSS and centrifuged (228×g/5min). After that the cell pellet was resuspended in a working solution (DAPI with 1:50 methanol) and incubated for 15 min at 37˚C in the dark. After centrifugation the pellet was resuspended in 25 μl HBSS. The cells were analysed under a fluorescence microscope (Nikon Eclipse TE 300).

Caspase-Glo 3/7 assay. The Caspase-Glo®3/7 assay (Promega, Mannheim, Germany) is a luminescent assay that measures the activities of the apoptotic key effector caspase-3 and-7 by cleaving the caspase3/7 substrate, which contains the tetrapeptide sequence DEVD. A total of 2×10^5 cells/ ml were treated with the LC_{50} concentration of the different extracts for different time points in duplicates. According to the manufacturer's instructions, 100 μl of Caspase-Glo®3/7 reagent were added to cells in each well of a white-walled 96-well NUNC-plate (Thermo Fisher Scientific, Vienna, Austria). After the incubation period of 2 h, the luminescence of each sample was measured directly by a plate-reading luminometer (Mediators Diagnostika GmbH, Vienna, Austria). Untreated cells were used as negative control.

Human Apoptosis Kit BD™ Cytometric Bead Array (CBA). The BD™ CBA Human Apoptosis Kit (BD Biosciences, Vienna) was used to quantitatively measure cleaved poly(ADP-ribose) polymerase (PARP), Bcl-2 and active caspase-3 protein in a single sample. The principle of the test is that there are three bead populations which have been coated with capture antibodies specific for PARP, Bcl-2 and caspase-3. MTC and HF-SAR cells were treated with *UNC* extracts for 4, 8, 12 and 24 hours. The cells were lysed with the Cell Lysis Buffer and the cell pellet was brought to a cell density of 2×10^6 cells/ml. According to the manufacturer's instructions, a standard curve was drawn. The instrumental analysis was performed with BD FACS Comp™ Software and BD Calibrite™ Beads.

Sub-G_1 peak and cell cycle. During apoptosis, calcium- and magnesium dependent nucleases are activated and cleave DNA in 200 bp. Since apoptotic cells have a lower DNA content, subsequent staining with a DNA binding dye will reveal these cells in the sub G_1 region of the cell cycle. A total of 5×10^5 cells were fixed in 70% ice-cold ethanol for 10 min at 4˚C. After washing, the cell pellet was resuspended in propidium iodide (PI)-staining buffer (50 μl/ml PI, RNAse A; Beckman Coulter, USA) and incubated for 15 min at 37˚C. The DNA content was analyzed using CellQuest Software with a FACSCalibur (Becton Dickinson, Heidelberg, Germany) flow cytometric system. In each sample, 10,000 fluorescent cells were counted.

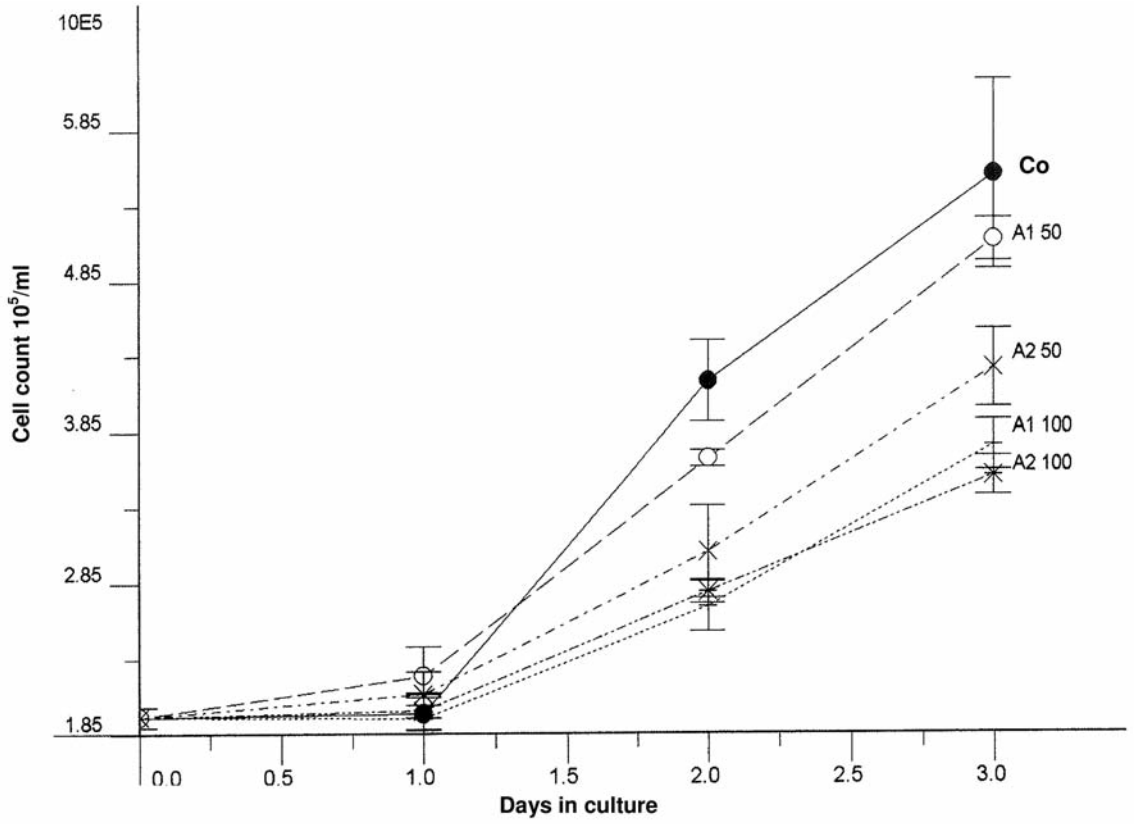

Figure 1. *Cell proliferation. Cell growth of MTC-SK cells was determined following treatment with different concentrations of isopteropodine (A1) and pteropodine (A2) from Uncaria tomentosa (UNC). 100 μM of A1 as well as 50-100 μM of A2 significantly inhibited cell proliferation of MTC-SK cells. Co represents untreated MTC-SK control cells. Error bars represent standard deviation σ.*

Statistical analysis. Statistical analysis was performed using the one-sided Student's *t*-test. *P*-values ≤0.05 were considered significant. All experimental results are expressed as mean values±standard error of the mean.

Results

Cell proliferation and WST-1 cell viability assay. Compared to controls, a 3-day treatment with extracts of *UNC* dose-dependently affected cell proliferation of MTC-SK cells in different ways. Isopteropodine (A1) at 100 μM as well as 50-100 μM of pteropodine (A2) significantly attenuated growth of MTC-SK cells (Figure 1). Similar results were obtained with concentrations up to 200 μM (not shown). In contrast, the alkaloid-poor methanolic extract (M) did not affect cell proliferation of MTC-SK cells (not shown). In the WST-1 assay, a concomitant decrease of mitochondrial dehydrogenase activity was observed following administration of the crude substances A1 and A2 (100-200 μM), whereas M (100-200 μM) did not show any effect on cell viability of MTC-SK cells (Figure 2A). Comparing the sensitivity of different tumor cell lines to *UNC* extracts, the MCF-7 cell line was noted to be equally sensitive as MTC-SK. Treatment with A1 produced antiproliferative effects in MTC-SK and the MCF-7 mammary carcinoma cell line (57.1% in both cell lines). A minor growth inhibition was found in KNA pheochromocytoma (36.0%) and in HBMC melanoma (13.0%). A2 treatment reduced proliferation in both MTC-SK and MCF-7 by 47.2%, in KNA by 26.4% and in HBMC by 30.4% (data not shown). Human skin fibroblasts (HF-SAR) served as non-malignant control cells. The same concentrations of M, A1 and A2 did not affect cell viability of HF-SAR cells (Figure 2B).

DAPI-stain. DAPI staining revealed morphological changes: cell shrinking, chromatin condensation and apoptotic bodies (arrow) were observed in A1- and A2-treated MTC cells while normal control cells were not impaired (Figure 3).

Sub-G_1 staining. The number of cells in the sub-G_1 population was quantified after treatment with A1 and A2 using flow cytometric analysis. After treatment with A1 and

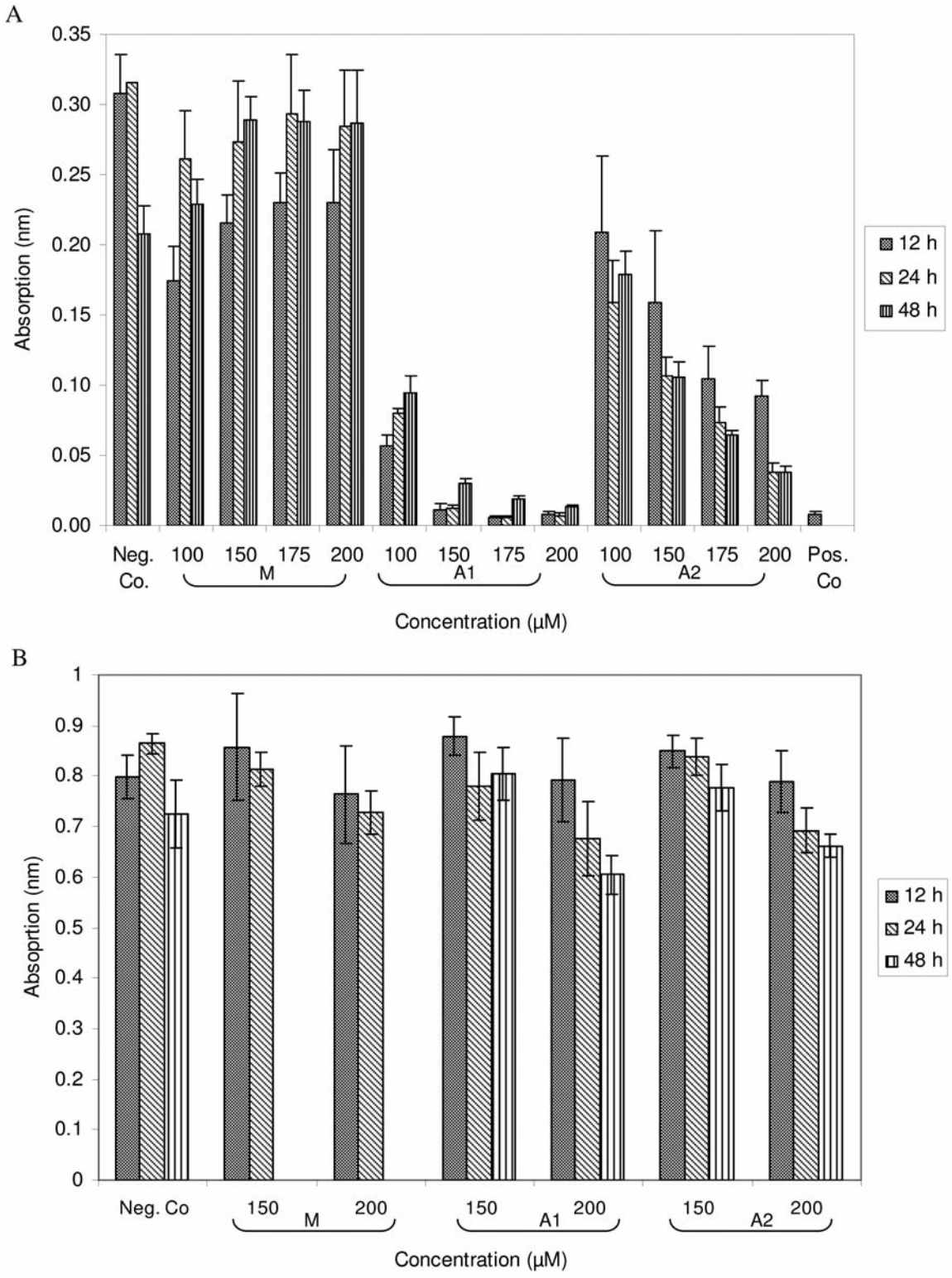

Figure 2. *Cell viability: A, MTC-SK cells were treated with different concentrations of the methanol extract (M) as well as the alkaloids isopteropodine (A1) and pteropodine (A2) from Uncaria tomentosa (UNC) and cell viability was measured. Treatment with 100-200 μM of the M fraction of UNC did not show any effect on cell viability of MTC-SK cells. In contrast, A1 and A2 of UNC (100 - 200 μM) significantly decreased the enzymatic activity of mitochondrial dehydrogenase of MTC-SK cells in the WST-1 assay. B, The same concentrations of M, A1 and A2 did not affect cell viability of normal human skin fibroblasts (HF-SAR cells) (positive control). Error bars represent s.e.m. All experiments were performed in triplicates.*

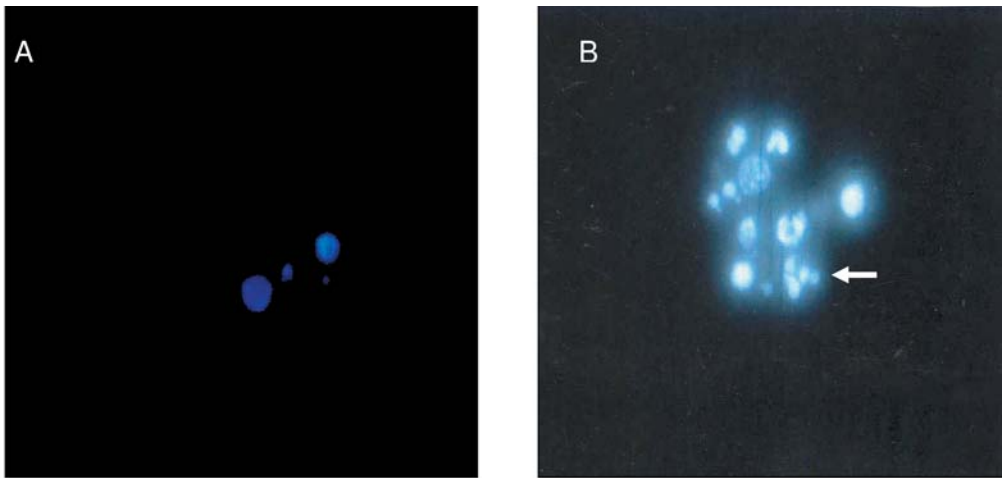

Figure 3. *DAPI-stain: A, Untreated control cells; B, MTC-SK cells were treated with A1 from Uncaria tomentosa (UNC), bright fluorescent chromatin indicates compact apoptotic cells.*

A2 for 24 h, the percentage of cells in the sub-G_1 fraction was 14% (Figure 4). After 48 h the percentage of cells in the sub-G_1 fraction was redoubled. However, a non significant increase in the percentage of cells in the sub-G_1 fraction was observed when cells were treated with the same concentration of M extract. A1 and A2 were able to induce a significant sub-G_1 peak; on the contratry, M extract did not enhance the number of cells in the sub-G_1 fraction.

Cell cycle arrest. To assess whether A1- and A2-induced cell growth suppression was mediated *via* alterations in the cell cycle, the cell cycle distribution was evaluated by flow cytometry. Since treatment with 200 μM M extract showed no affects on cell growth and viability, not surprisingly, M extract brought about no significant alterations of the cell cycle. A1 caused a significant decrease in the G_2/M-phase cell population (17.76% in control *versus* 9.83% after 12 h and 10.02% after 24 h and 4.44% after 48 h in cells treated with 200 μM A1 (Figure 4). A2 caused after a significant decrease in the G_1-phase cell population (56.68% in control *versus* 22.47% after 12 h, 43.17% after 24 h and 36.74% after 48 h.

Cleaved caspase-3 and -7. To investigate whether caspase-3 was activated in MTC cells treated with *UNC*, caspase activity was measured using Caspase-Glo 3/7 assay. As shown in Figure 5 A, A1 and A2 extracts increased caspase-3 like acitivity in a dose and time dependent manner. Maximum A1 activity was seen at 24 h and A2 activity at 48 h. On the contrary, M-extract of *UNC* did not give rise to cleaved caspase-3. These results demonstrate that alkaloids of UNC induced apoptosis in MTC-SK cells in a caspase-3/7 dependent manner, but not in normal cells.

Human Apoptosis Kit BD™ (CBA). To measure the A1- and A2-induced apoptosis, a CBA was carried out. Figure 6 shows that there was a significant increase in protein levels of cleaved PARP and active caspase-3 in a dose- and time-dependent manner on treatment with 200 μM A1 and A2. Whereas the Bcl-2 protein level did not change (Figure 6) as determined by BD™ Human Apoptosis Kit. MTC cells treated with 200 μM A1 showed an increase of cleaved caspase-3 after 24 h. The level of cleaved PARP had a 3-fold increase in MTC cells treated with 200 μM A1 after 8 h compared to the control (Figure 6A). Also treatment with 200 μM A2 gave a significant increase of cleaved caspase-3 and PARP after 8 h, whereas after 12 h and 24 h, the amount of cleaved caspase-3 and PARP decreased compared to the control. MTC cells treated with M extract showed no cleaved caspase-3 nor cleavage of PARP, furthermore, no changes in the Bcl-2 expression were found (data not shown). Normal fibroblasts did not show any changes relating to cleaved caspase, PARP or Bcl-2 (data not shown).

Discussion

At the time of diagnosis, more than 25% of patients with MTC have distant metastases. In addition, MTC is known to be relatively insensitive to radiation therapy as well as to chemotherapy and, to date, no effective treatment for patients with advanced MTC is available. Resistance to chemotherapy was attributed to the expression of the multidrug resistance *mdr1* gene (32). Cell lines of MTC are critical for studies of anticancer agents. However, the development of MTC cell lines has proven difficult in the past. For decades, only one continuous MTC-cell line, TT, was available (33). In our previous attempts to culture MTC,

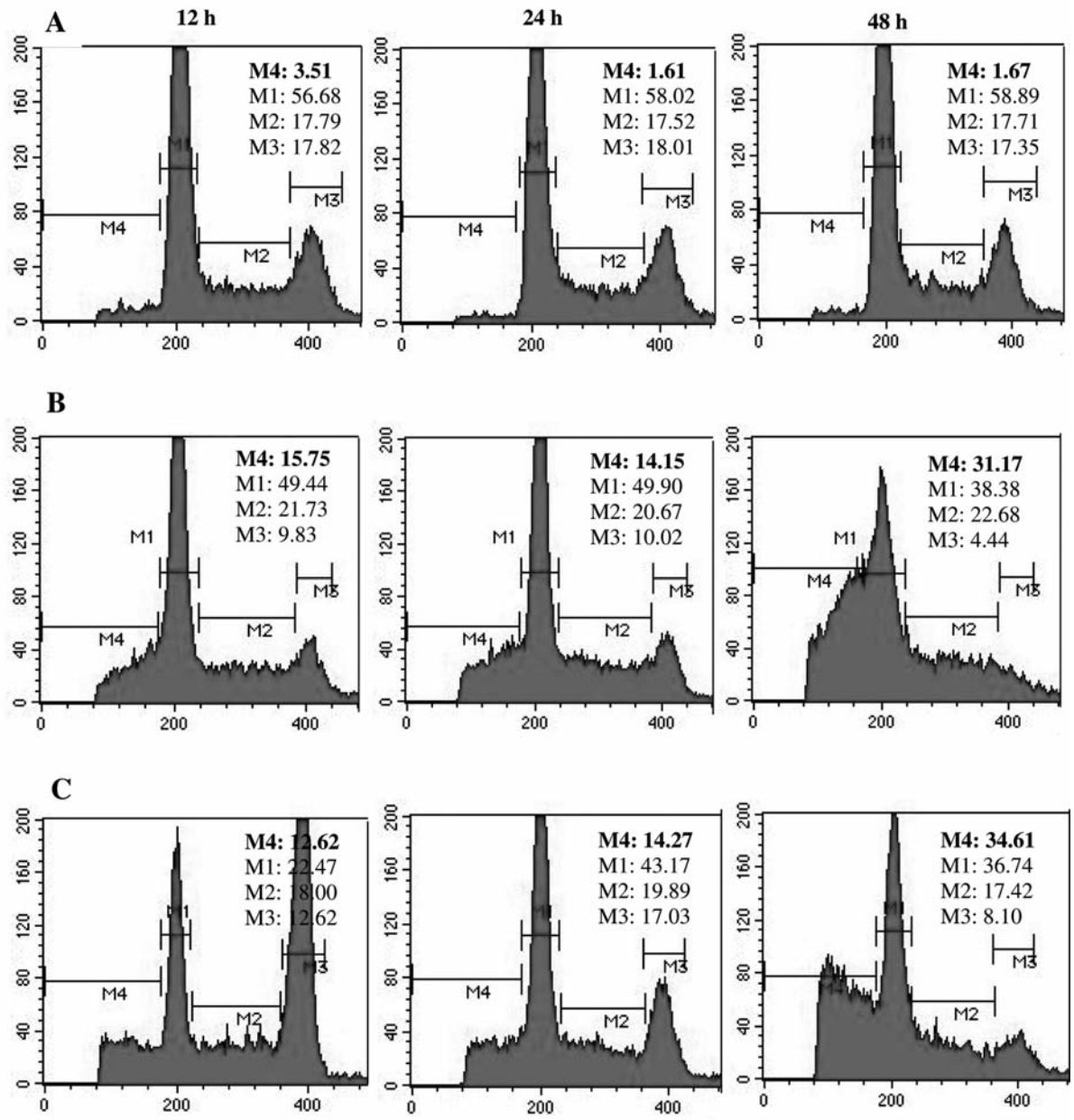

Figure 4. *DNA-Cycle: The sub-G1 (M4) ratio in MTC-SK cells treated with Uncaria tomentosa (UNC) was examined using flow cytometry. Cells were harvested after 12 h, 24 h and 48 h then they were fixed stained and analyzed for DNA content. The distribution and percentage of cells in sub-G1 phase (M4), G1 (M1), S (M2) and G2/M (M3) phase of the cell cycle are indicated. The sub-G1 peak displays cells with a lower DNA content. A) Treatment of MTC-SK cells with 200 μM M fraction from UNC did not affect the cell cycle after 12, 24 and 48 h. Furthermore, no apoptotic signs became visible. B) Following administration of 200 μM of A1 a sub-G1 peak was detectable in MTC-SK cells after 12 h, which was enhanced after 48 h. C) Cells were treated with 200 μM A2 of UNC, a sub-G1 peak was detectable in MTC-SK cells after 12 h and increased after 48 h.*

we have established eight continuous cell lines, including MTC-SK (34). Each MTC cell line was tumorigenous and showed an increased expression of the anti-apoptotic protein Bcl-2, allowing the tumor cells to survive.

In the present study we showed that *UNC* affected cell growth, viability, and apoptosis of MTC-SK cells in different ways. Whereas the alkaloids isopteropodine (A1) and pteropodine (A2) significantly inhibited cell growth and viability of MTC-SK cells, the methanol extract (M) did not affect cell proliferation nor mitochondrial dehydrogenase activity.

In initial studies, we observed that the M extract of *UNC* did not affect growth parameters of MTC-SK cells

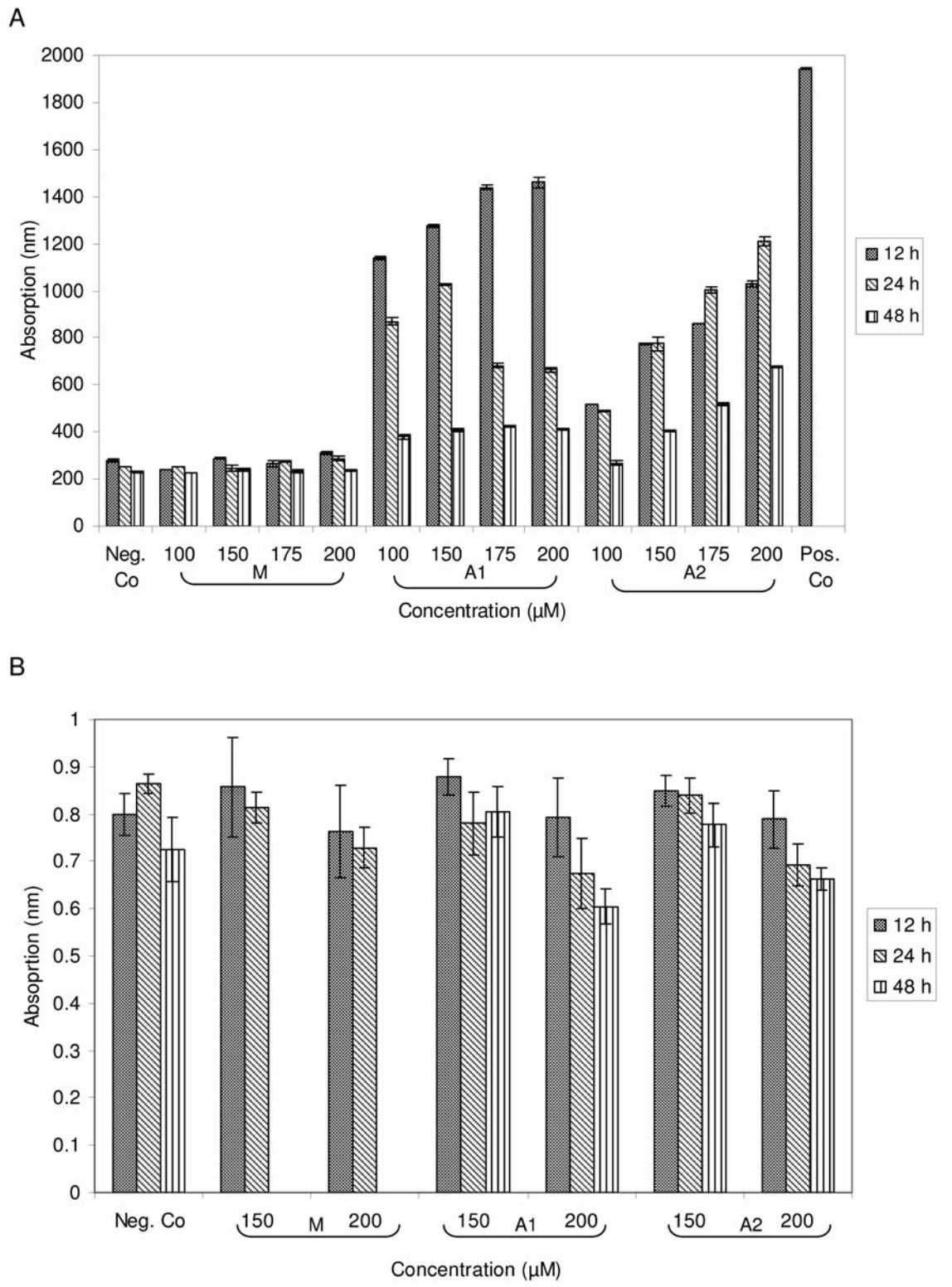

Figure 5. *Caspase-3 activity was examined following treatment with different concentrations of the alkaloid-poor methanol fraction (M), as well as the alkaloids isopteropodine (A1) and pteropodine (A2) from Uncaria tomentosa (UNC). A, MTC-SK cells treated with 100-200 µM of the M fraction of UNC did not show cleaved caspase-3 increases. In contrast, a significant increase in caspase-3 activity was observed in cells treated with 100-200 µM of A1 of UNC. Cells treated with A2 (100-200 µM) displayed less cleaved caspase-3 increase than cells treated with A1. B, Neither treatment with the M fraction nor with A1 and A2 (150-200 µM) affected caspase-3 activity of HF-SAR cells.*

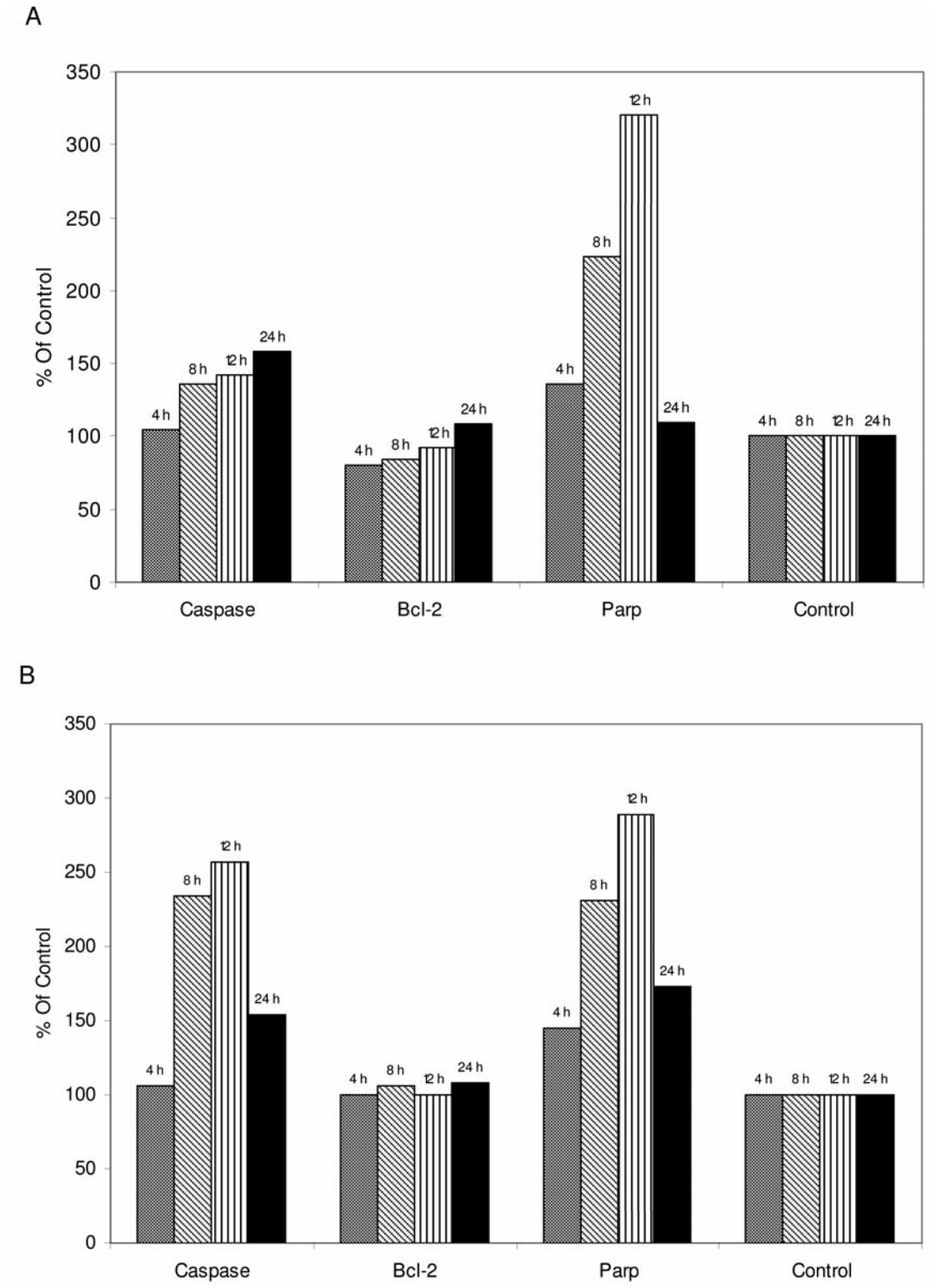

Figure 6. *Cytometric Bead Array measurement: The simultaneous measurement of cleaved caspase-3, Bcl-2 and PARP was performed with the CBA kit. Following treatment of MTC-SK cells with 200 μM A1 (A) and A2 (B) of UNC, an increase of cleaved caspase-3 as well as an enhancement of PARP was observed. However, in MTC-SK cells treated with A1, the highest cleaved caspase-3 amount was detected after 24 h, whereas a high amount of cleaved caspase-3 in cells treated with A2 was already detected after 8 h. No significant change in Bcl-2 expression was detected. Normal fibroblasts showed no cleaved caspase-3, no enhancement of PARP and no change in Bcl-2 expression (data not shown).*

(21) Thus, in the subsequent experiments this extract could be used as positive control for drug actions. In recent years, an increasing number of studies described extracts (*e.g.* alkaloids, terpenes, flavonoids, quinovic acid glycosides) of *UNC* with potential antiviral, anti-inflammatory, anti-rheumatic and anticancer properties (35-37). The induction of apoptosis by several organic extracts of *UNC* in human promyelocytic leukemia (HL-60) cells was described by Cheng *et al*. (38) who found that *n*-hexane (CC-H), ethyl acetate (CC-EA) and *n*-butanol extracts (CC-B) had a greater anticancer effect on HL-60 cells than those extracted with methanol (CC-M). This is in line with our results, which showed that the methanol extract (M) also did not affect cell growth of MTC-SK cells. Furthermore, this group detected that CC-EA induced DNA fragmentation in HL-60 cells in a more pronounced manner than did the other extracts (38). Extracts from *UNC* have also been shown to be effective against tumors of the central nervous system. For example, mitraphylline, a pentacyclic oxindole alkaloid of *UNC*, has been found to exert strong antiproliferative effects on two human brain tumor cell lines (neuroblastoma SKN-BE(2) and malignant glioma GAMG (39). Our study clearly indicates that specific alkaloids of *UNC* also act as strong inhibitors of proliferation of chemorestistant neuroendocrine tumors.

The alkaloids of *UNC* produced an antiproliferative effect in MTC-SK cells by G_2 arrest. Previous reports showed that *UNC* blocked G_2/M transition of tumour cell proliferation and inflammatory responses (40, 23). We found that Bcl-2 overexpression was not influenced and did not prevent the MTC cells from programmed cell death. The effect of A1 and A2 of *UNC* showed clearly specific caspase-3-dependent apoptosis in MTC cells, whereas the M extract of the same plant did not show any effect. Moreover, no loss of viability on treatment with the alkaloids A1, A2 and with M extract was detected in normal human fibroblasts. Summarizing, A1 and A2 of *UNC* seem to be excellent candidates for further evaluation as effective therapeutic agents through reduction of proliferation and induction of apoptosis in MTC cells.

Acknowledgements

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