Abstract. Background: Medullary thyroid carcinoma (MTC) is a calcitonin-producing tumor of the thyroid arising from the parafollicular C-cells. MTC is poorly responsive to chemotherapy and radiotherapy, hence the only effective therapy is surgery. Based on this fact, alternative strategies have been sought. Materials and Methods: The effects of Cautleya gracilis (Smith) Dandy were investigated for the first time in three human MTC cell lines and in MTC-transplanted mice. Proliferation and viability were quantified by cell counting, WST-1 tests, and ATP luminescent cell viability assays. Apoptosis was studied by DAPI staining, flow cytometry and luminescent assays for caspases 3/7, 8 and 9. Results: A dose-dependent reduction of proliferation and an induction of apoptosis were found in all MTC cell lines, while normal fibroblasts were not impaired. Similar tumor inhibition was seen in heterotransplanted mice. Conclusion: Our in vitro and in vivo findings suggest a new potential clinical effect of Cautleya.

Medullary thyroid carcinoma (MTC) is a neuroendocrine tumor arising from the parafollicular C-cells of the thyroid gland. In 70-80% of cases, MTC occurs sporadically. The other 20-30% are inherited and may occur in three distinct clinical settings: as familial MTC without associated endocrinopathies, or combined with other endocrinopathies as multiple endocrine neoplasia type 2A or 2B with autosomal dominant inheritance (1-3). At the time of diagnosis, more than 25% of patients have distant metastases. Despite active study, neither chemotherapeutic agents nor irradiation have proved to be markedly effective in MTC therapy. Apoptosis and proliferation are directly correlated with bcl-2 expression (4). Resistance to chemotherapy was attributed to the expression of the multidrug resistance mdr1 gene (5).

Well-characterized human cell lines derived from MTCs have been very rare. For decades, only one continuous cell line, TT, was available (6). The TT cell line was derived from a hereditary MTC with an exon 11, codon 634 mutation (7). We have established eight continuous MTC cell lines (8–10), including MTC-SK (11) and SINJ (12) for deep investigation (13). MTC-SK and SINJ were derived from sporadic forms of MTC. Each of our MTC cell lines showed an increased expression of the anti-apoptotic protein Bcl-2, allowing the tumor cells to survive. Recently, oriental medicinal herbs with anticancer activity have come into the spotlight as complementary or alternative medicines (14). Thus far, several plant-derived drugs have received Food and Drug Administration (FDA) approval for commercial production. Examples of antineoplastic botanical drugs are camptothecin, vincristine and taxol (14).

In a preceding screening using MTT cell proliferation tests (Roche Diagnostics, Vienna, Austria) in HeLa cells, Cautleya...
"C. gracilis" (Smith) Dandy was selected for the present study (personal communication S Sturm, Institute of Pharmacy, Innsbruck). C. gracilis, sometimes called "hardy ginger", is a native of cool forests of the eastern Himalayas, belonging to the family of Zingiberaceae. The ethnobotanical activity of C. gracilis is unclear and C. gracilis extracts have never been investigated in the treatment of MTC. Therefore, as a first step to provide scientific evidence for anticancer activity, different fractions of C. gracilis were investigated in three human MTC cell lines and in xenotransplanted MTC mice.

The aim of the present study was to assess the in vitro and in vivo activity of C. gracilis extracts in chemoresistant MTC.

Materials and Methods

Plant material. The plant material (aerial parts) was provided by E. Stöger (Obendorf, Austria). The dried aerial parts of Cautleya gracilis (Smith) Dandy, Zingiberaceae, were ground and successively extracted with petroleum ether (PE) (CG-1), dichloromethane (DCM) (CG-2), and ethyl acetate (EtOAc) (CG-3) in a Soxhlet apparatus. The solvent was evaporated under reduced pressure. The dry extracts were then redissolved in dimethyl sulfoxide (DMSO; Sigma, Vienna, Austria) at a concentration of 10 mg/ml and stored at -20°C. The positive control camptothecin (CPT) (Sigma) was also dissolved in DMSO and stored at 4°C in 1 mM stock solution.

Cell lines and cell culture. The human MTC cell lines MTC-SK (11) and SINJ (12), and the normal human skin fibroblast cell line HF-SAR, were established in our laboratory. The human MTC cell line TT (6) was purchased from the European Collection of Cell Cultures (ECACC, Porton Down, UK; Cat. No. 92050721). MTC-SK and SINJ cells were maintained in Ham’s F-12 medium (Biowhittaker, Verviers, Belgium) containing 10% fetal bovine serum (PAA Laboratories, Exon, PA, USA) at 37°C, 5% CO₂. TT cultures (ECACC, Porton Down, UK; Cat. No. 92050721). MTC-SK and SINJ cells were maintained in Ham’s F-12 medium (Biowhittaker, Verviers, Belgium) containing 10% fetal bovine serum (PAA Laboratories, Exon, PA, USA) at 37°C, 5% CO₂. TT and HF-SAR cells were cultured in 1:1 Ham’s F-12: M-199 medium, supplemented with 10% fetal bovine serum, at 37°C, 5% CO₂. All cell lines were Mycoplasma-free.

Cell counting. MTC-cells were seeded at a density of 2x10⁵ cells/ml into 24-well plates (Sarstedt, Wn, Neudorf, Austria) and incubated for 24, 48 or 72 h in completed Ham’s F12 medium with DMSO (control) or supplemented with 10, 25, or 50 μg/ml of C. gracilis extracts. The cell clusters were pipetted into single cells and counted automatically (Casy-1 Cell Counter and Analyzer, Schärfe System, Reutlingen, Germany). Each sample was measured 3 times; the cell counter calculated the mean value and standard deviation (S.D.).

WST-1 cell proliferation assay. Cell proliferation and viability were quantified using reagent WST-1 Cell Proliferation Reagent (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulphonate) (Roche Diagnostics, Vienna, Austria) according to the manufacturer’s protocol. This method is based on the ability of viable cells to metabolize tetrazolium salt WST-1 to formazan by mitochondrial dehydrogenases. MTC-SK and SINJ cell suspension were seeded at a density of 2x10⁵ cells/ml in 24-well plates (Sarstedt). After 24, 48 or 72 h treatment with different concentrations of solvent extracts or DMSO, cells were pipetted carefully into single cells and transferred into 96-well plates; 10 μl WST-1 labeling mixture per well was then added per well. After incubation at 37°C for 2 hours, the samples were quantified spectrophotometrically by measuring the absorbance of the formazan product at 450 nm with an ELISA plate reader. For TT and HF-SAR adherent cells, cells were seeded directly into 96-well plates at a density of 1x10⁵ cells/ml. After adherence to plates, the cells were treated with extracts for 24, 48 and 72 h and measured as above. Each sample was tested in 6 replicates; means and S.D. were calculated.

ATP luminescent cell viability assay. This assay was performed using the CellTitert-Glo luminescent cell viability assay kit (Promega, Madison, WI, USA Cat # G7570). One ml of cell was seeded into a 24-well plate at a density of 2x10⁵ cells/ml and incubated in completed Ham’s F12 medium with 10, 25, or 50 μg/ml extracts, or 5 μl DMSO negative control for 4 h at 37°C. After equilibrating to room temperature for 30 min, MTC-SK cells were pipetted carefully into single cells. Twenty-μl of each sample were added to white-walled 96-well plates (Nunc, Roskilde, Denmark), then a 1:1 ratio of Caspase-Glo3/7 reagent volume to sample volume was added into the same well. After mixing and incubating at room temperature for 10 min, all samples were measured in a plate-reading luminometer (Mediators PhL, Mediators Diagnostika, Vienna, Austria) as directed by the manufacturer. Each treatment was measured for 3 parallel samples, and mean value and S.D. were calculated.

DAPI (4’6’-diamidino-2 phenylindole) staining. TT cells were seeded on FlexiPerm slides (Kendro, Vienna, Austria) overnight for adherence and then treated with CG-1 at a concentration of 25 μg/ml for 4, 12 and 24 h. The slides with TT cells were washed with Dulbecco’s-phosphate-buffered saline lacking Ca²⁺ and Mg²⁺ (PBSA) and stained with 0.5 μg/ml DAPI (Sigma) in methanol for 30 min at room temperature. Cells were viewed using an inverted phase-contrast fluorescence microscope with ultraviolet (UV) excitation at 300-500 nm (Nikon Eclipse TE300, Tokyo, Japan). Cells with nuclei containing clearly condensed chromatin or cells with fragmented nuclei were scored as apoptotic.

FACS analysis. To confirm apoptosis, active caspase-3 staining was performed using the FITC-conjugated Monoclonal Active Caspase-3 Antibody Apoptosis Kit (BD Bioscience, Vienna, Austria; Cat #550480) according to the manufacturer’s instructions: Approximately 1x10⁵ cells were treated with different concentrations of CG-1 for 24 h, washed in cold PBSA, fixed and permeabilized using 500 μl Cytofix/Cytperm™ solution for 20 min on ice. Cells were washed and resuspended in 100 μl Perm/Wash™ buffer containing 20 μl PE-conjugated monoclonal rabbit anti-active caspase-3 antibody at room temperature for 30 min. After incubation, cells were washed in Perm/Wash™ buffer and analysed with FACSscan (BD, Heidelberg, Germany).

TUNEL assay. Cells were detected using the In Situ Cell Death Detection Kit, Fluorescein (Roche, Lewes, UK; Cat #11684795910) according to the manufacturer’s instructions. Briefly, cells were treated with CG-1, CG-2 or CG-3 for 24 h, washed with PBSA and harvested. The cells were fixed with freshly prepared 4% paraformaldehyde for 60 min at room temperature and placed on ice for 2 min with 0.1% Triton® X-100 solution (Serva, Heidelberg, Germany). Intracellular DNA fragments were then labeled by exposing the cells to TUNEL...
reaction mixture for 1 h at 37°C, in a humidified atmosphere and protected from light. After washing with PBSA twice, cells were transferred to a slide and analyzed under a fluorescence microscope (Nikon Eclipse TE300, Tokyo, Japan).

Caspase 3/7 activity assay. Caspase 3/7 activities were measured with a homogenous luminescent assay kit (Promega, Madison, WI, USA; Cat # G8091) according to the manufacturer’s instructions. One ml MTC-SK cells was seeded into 24-well plates at a density of 2x10^5 and incubated in completed Ham’s F12 medium with 10, 25, or 50 μg/ml substances; 5 mM CPT positive control, or 5μl DMSO negative control for 4 h at 37°C. After equilibrating to room temperature for 30 min, MTC-SK cells were carefully pipetted into single cells; 25 μl of the total volume of each sample was added into white-walled 96-well plates (Nunc), then a 1:1 ratio of Caspase-Glo 3/7 reagent volume to sample volume was added into the same well. After gently mixing the contents of wells on a plate shaker for 30 s, they were incubated at room temperature for 1 h and finally measured for luminescence of each sample in a plate-reading luminometer (Mediators PhL) as directed by the manufacturer. Each treatment was measured in 3 parallel samples, and the mean value and S.D. were calculated.

Time curve of caspase-8, caspase-9, and caspase-3/7 activity. Caspase-8, caspase-9 and caspase-3/7 activities were measured using Promega luminescent assay kits (Cat # G8201, Cat # G8211, and Cat # G8091, respectively). Briefly, MTC-SK cells were seeded at a density of 2x10^5/ml and 25 μg/ml of CG-1, CG-2, CG-3 or DMSO solvent were added for 0, 1, 4, 8, 12, and 24 h. Each sample was pipetted carefully into single cells, then transferred into three 96-well white-walled plates (Nunc) and measured for its caspase-8, caspase-9 and caspase-3/7 activity according to the manufacturer’s instructions. Each treatment was measured in 3 parallel samples, and the mean value and S.D. were calculated.

In addition, the effect of the specific caspase-3 inhibitor Ac-DEVD-CHO (Promega, Madison, WI, USA, Cat # G5961) on MTC-SK cells treated with 10 μg/ml CG-2 was determined. Thereafter, MTC-SK cells were seeded into 24-well plates at a density of 2x10^5 cells/ml. One group had been co-treated with specific caspase-3 inhibitor Ac-DEVD-CHO fifteen minutes before CG-2 or camptothecin were added, whereas the other group did not receive the specific inhibitor. Caspase-3/7 activity was measured at 4, 7, 9, 14, 16, 22 and 24 h and the activity of Ac-DEVD-CHO at 4, 9, 14 and 22 h using the Promega luminescent assay kit according to the manufacturer’s instructions, as described above.

Transmission electron microscopy. MTC-SK and HF-SAR cells were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, on ice (Plano, Wetzlar, Germany), postfixed in 1% osmium tetroxide (SIGMA) in 0.1 M cacodylate buffer and processed by modified routine methods. Photographs were taken with a FEI Technai 12 equipped with a Gatan CCD Camera BioScan.

Treatment of MTC xenografts in SCID mice with CG-2. Female SCID mice about 6 weeks old (Department of Laboratory Animal Science and Genetics, Medical University of Vienna, Himberg, Austria) were injected intratumorally with a) 10 μg/100 μl CG-2 dissolved in DMSO/ PBSA per tumor for 5 mice , b) DMSO/ PBSA (control 1) for 5 mice, and c) PBSA only (control 2) for 4 mice. The observation period was 21 days.

Results

Antiproliferative activity of C. gracilis extracts in human MTC cell line MTC-SK. First, to ascertain anticancer activity, all three extracts of C. gracilis, CG-1, CG-2 and CG-3, were evaluated for their antiproliferative activities in the human medullary carcinoma cell line MTC-SK by cell counting. At a density of 25 μg/ml, all these extracts prominently suppressed proliferation in MTC-SK cells. As shown in Figure 1A, after 72 h treatment, cells added with DMSO solvent reached a density of 5.0x10^5 cells/ml from the starting concentration of 2.35x10^5 cells/ml; however, those cells with C. gracilis extracts only reached a density of less than 2.5x10^5 cells/ml with the same starting density.

To further test the antiproliferative effect, we used the WST-1 cell proliferation reagent. Within 48 h of exposure, all the extracts had the potency to reduce cell viability in a dose-dependent manner. At a concentration of 50 μg/ml, more than 90% of cells lost their viability within 48 h treatment. The IC50 values of CG-1(P/PE extract), CG-2 (DCM extract) and CG-3 (EtOAc extract) were approximately 20, 18 and 15 μg/ml, respectively (Figure 1B).

Moreover, we used an even more sensitive method, the ATP luminescent cell viability assay based on the amount of ATP, to quantify the cell viability changes within a short-term treatment. As shown in Figure 1C, even with 4 h exposure, high concentrations of C. gracilis extracts could also reduce MTC-SK cell viability in a dose-dependent manner. At a concentration of 50 μg/ml, the percentage of remaining cell viability under treatment with CG-1, CG-2, and CG-3 for 4 h was 29.8%, 50.4% and 42.8%, respectively.

Effects of C. gracilis on multicellular aggregation and cell morphology of MTC-SK cells. As reported previously by our group (20), MTC-SK cells exhibited an ability to survive and proliferate in the aggregate form. It is believed that the ability of cell populations to survive in the aggregate form is a potential index for cell tumorigenicity, which might also be a reason for drug and radiation resistance in cancer (5-7). Thus, we aimed to study the effect of C. gracilis extracts on intact spheroids of MTC-SK medullary carcinoma cells. Multicellular aggregates were formed by the MTC-SK cells and cultured in 10% FBS for 24 h. At this time, the intact spheroids were treated with and without C. gracilis extracts. After a 24-h treatment, the spheroids were observed under light microscopy and photographed. Figure 2A (a) shows MTC-SK cells grown as multicellular tumor spheroids without the addition of extracts, whereas Figure 2A (b-j) shows the effect of different concentrations of C. gracilis
Extracts on the previously intact spheroids. Cellular adhesion in the treated cells was significantly disrupted.

Extracts of C. gracilis induced apoptosis in MTC-SK cells. To investigate whether the extract-induced inhibition of cell proliferation was associated with cell apoptosis, TUNEL assay (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling; Roche, Lewes, UK, cat # 11684795910) was performed to detect apoptotic morphologic changes. MTC-SK cells were incubated with 25 μg/ml CG-1, CG-2, CG-3 or DMSO control for 24 h and stained with fluorescein-12-UTP. As shown in Figure 3, CG-1-, CG-2-, and CG-3-treated MTC-SK cells showed green fluorescence, indicating the fragmented DNA in apoptotic cells, whereas the DMSO control was negative for the staining.

To further confirm the induction of apoptosis, FACS analysis with active caspase-3 antibody staining was performed in CG-1-treated MTC-SK cells as shown in Figure 4A. With 24 h treatment, CG-1 increased the proportion of activated forms of caspase-3-positive cells from 7% in control cells to 43.67%, 87.94%, and 90.77% in 10, 25, and 50 μg/ml CG-1-treated cells, respectively.

C. gracilis extracts induced activation of caspases. To confirm further the apoptosis and determine whether caspase pathways were involved in this process, the activities of activated forms of caspase-3/7, caspase-8, and caspase-9 were detected using an in vitro luminescence-based assay. Firstly, the activity of caspase-3/7, which are the final effector caspases in apoptosis, was quantified at different concentrations of CG-1, CG-2 and CG-3 treated MTC-SK cells. As shown in Figure 4B, these three extracts all increased active caspase-3 activity in a dose-dependent manner. After 4 h exposure at a concentration of 50 μg/ml, the caspase-3 activity of CG-1-, CG-2- and CG-3-treated cells was 6.8-, 8.5- and 7.6-fold more than in control cells. In addition, the caspase-3 activity of MTC-SK cells and the cell viability were found to be inversely correlated (compare Figure 4A to 1C), suggesting that C. gracilis inhibited the growth of MTC-SK cells, at least in part, by inducing apoptosis. The specific caspase-3/7 inhibitor Ac-DEVD-CHO confirmed the results by blocking the caspase-3/7 activity totally (Figure 5).

Besides caspase-3/7 effector caspases, the activation of initiator caspases, caspase-8 and caspase-9 was also measured. We observed that in 25 μg/ml C. gracilis-treated cells, caspase-8, -9, and -3/7 activities significantly increased compared to control cells. Caspase-8, -9, and -3/7 peaked after about 8 h of treatment (Figure 6 A, B, C). Data of normal cells are not shown.

Different effects of C. gracilis on human MTC cells compared to normal fibroblasts. From previous results, it was deduced that the C. gracilis extracts had strong anticancer effects on...
MTC-SK cells, though it was unclear whether *C. gracilis* acted against other cancer cell lines as well. To study generalized applicability, we tested two other medullary carcinoma cell lines, SINJ and TT, compared to the normal human fibroblast cell line HF-SAR for their sensitivity to CG after 48 h treatment (Figure 7 A, B, C). Using the concentration of *C. gracilis* required to kill 50% of the cells (IC$_{50}$) as a measure of sensitivity, we detected variable sensitivity among cell lines, with MTC-SK being most sensitive (IC$_{50}$ = 18 to 23 μg/ml), and SINJ and TT having an intermediate sensitivity (IC$_{50}$ = 22 to 40 μg/ml and 22 to 40 μg/ml respectively). In contrast, the fibroblast cell line HF-SAR was more resistant (IC$_{50}$ > 50 μg/ml). The IC$_{50}$ difference of CG on MTC cells compared to fibroblasts suggested that *C. gracilis* displayed special anticancer activity in MTC-SK, SINJ and TT cells, therefore suggesting it to be a potential chemotherapeutic agent for the control of MTC.

**Electron microscopy.** Ultrastructural changes in the tumor cells were observed after 24 h treatment with *C. gracilis* extracts. The MTC cells showed condensation of chromatin at the periphery of the nuclear membrane. The plasma membrane formed many blebs and protrusions (Figure 8A). The control cells, normal human skin fibroblasts, showed no ultrastructural modification under treatment with *C. gracilis* extracts. The structures of the cell membrane, nucleus and organelles were normal, without any apoptotic changes (Figure 8B).

**Treatment of MTC xenografts in SCID mice with CG-2.** Tumor growth was followed in each experimental group. Similar to the *in vitro* results, a tendency toward tumor inhibition was found in all CG-2-treated mice. No effects were measured in the two control groups: the growth of the untreated tumors was not inhibited, as measured by tumor diameter (data not shown).
Discussion

In this study, we present a first report on the anticancer activity of C. gracilis extracts on human medullary carcinoma cells. All three extracts from C. gracilis had a strong inhibitory effect on tumor cell growth, disrupting the tumor spheroids, and induced tumor cell apoptosis. CG-1, CG-2 and CG-3 inhibited MTC-SK, SINJ and TT medullary thyroid cells growth in a dose-dependent manner. The growth suppressive function of C. gracilis on MTC cells was very strong; even with a 4 h treatment, cell viability was largely reduced (Figure 1C). The IC₅₀ in the medullary cell lines MTC-SK, SINJ and TT was between 18-40 μg/ml. In contrast, in normal fibroblasts, at a concentration lower than 50 μg/ml, those extracts had almost no suppressive effect. Almost all conventional anticancer drugs are primarily effective against rapidly dividing cells. The strong proliferative effect and the IC₅₀ difference between MTC cell lines and the slowly dividing fibroblasts suggested that C. gracilis could be a candidate for effective chemotherapeutic agents, especially in MTC.

Chemotherapeutic drugs are considered to kill tumor cells by activating a cascade of events resulting in apoptosis. In agreement with this line of thought, we provided evidence that C. gracilis extracts were able to induce apoptosis with the classic feature of apoptosis including initiator and effector caspase activation and internucleosomal DNA fragmentation. Bcl-2 overexpression did not prevent apoptosis. In addition, further analyses revealed that the decrease of cell viability with the treatment was associated with the induction of apoptosis in MTC-SK cells (compare Figure 1C with Figure 4A, r=0.9, 0.97 and 0.98, respectively), suggesting that cell death could be a main reason for the decrease in cell viability.

Moreover, we tried to determine the caspase activation pathway in C. gracilis-treated MTC-SK cells. At present, the receptor-binding and mitochondrial-mediated apoptosis pathways are two major modes of caspase activation that have come to light. In this study, we found that both the initiator caspase-8, which is mainly involved in the external pathway, and the internal pathway-related initiator caspase-9 were activated. Caspase-3 and caspase-9 activation implied the involvement of the mitochondrial pathway in C. gracilis-induced apoptosis. Caspase-8 activation was induced by extrinsic receptor binding and followed by direct activation of caspase-3 or crosstalk with the mitochondrial mediated pathway by cleavage of Bid.
The data suggest the possible involvement of an extrinsic pathway for the induction of apoptosis by *C. gracilis*. However, more work is required to delineate the relative contribution of this pathway to apoptosis execution.

The *C. gracilis* extracts also disrupted the intact multicellular tumor spheroids in MTC-SK and SINJ cells. The ability of tumor cell populations to survive in the aggregated form is a potential index for tumorigenicity.
which might be a reason for drug and radiation resistance in cancer (15-17). Both MTC-SK and SINJ cell lines exhibit the phenomenon of growth \textit{in vitro} as multicellular aggregates. In our experience, only about 1-5\% of MTC-cell lines grow adherently \textit{in vitro}, while the majority form densely packed spheroidal aggregates. This phenomenon might also account for their chemoresistance. Multicellular resistance reflects a relative intrinsic phenotype and a disruption should actually contribute to growth inhibition. Identical observations were described in previous studies on other bioactive agents, including extracts of \textit{Stemona tuberosa} Lour (18-20).

The \textit{in vivo} experiment with MTC-xenografted mice resulted in growth retardation of the tumors in the CG-2-treated group. In contrast, the control groups with untreated xenografts showed unimpaired tumor progression. As this pilot experiment involved only a small number of mice, statistical analyses are in progress with a larger number of animals.

Compared with the antitumor effects of other bioactive agents, derived from \textit{Stemona tuberosa} Lour and other plants (18-20), the effects of \textit{C. gracilis} on proliferation, morphology and apoptotic rates of MTC were clearly stronger: even a very low dose of \textit{C. gracilis} (10 μg/ ml) was effective in MTC cells, while Stemona effects became manifest from concentrations of 25 μg/ ml upwards. Summarizing, the novel bioactive compounds of \textit{C. gracilis} seem to be good candidates for further evaluation as effective therapeutic agents acting through reduction of proliferation and induction of apoptosis.

**Acknowledgements**

This investigation was supported by the Austrian Cancer Aid/ Styria (EF 01/2004). We wish to thank Ms. Eugenia Lamont for critically reviewing the manuscript.

**References**


