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Activity of Novel Plant Extracts Against Medullary Thyroid Carcinoma Cells

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Abstract. *Background:Medullary thyroid carcinoma (MTC) is a rare calcitonin-producing tumor, derived from the parafollicular C-cells of the thyroid. MTC is known to be relatively insensitive to conventional chemotherapy. Materials and Methods: Eight cell lines were established from MTCs; each showed an up-regulation of Bcl-2. We investigated ten agents from plants of the genera Stemona (Stemonaceae), Aglaia (Meliaceae) and Artemisia (Asteraceae) for their effects on proliferation and apoptotic rates. Extracts have been used in traditional Chinese medicine; however, no experience on their effects on medullary thyroid carcinomas has been reported so far. Growth kinetics and viability were examined using the Casy-1-Cell Counter & Analyzer and the WST-1 - based cytotoxicity assay. Apoptosis was studied by DAPI staining, by measurement of caspase-3 activity and Bcl-2 expression. Results: A strong antiproliferative effect was recognized in each Aglaia species and with Artesunate, whereas an enhancement of apoptosis was provoked particularly by Stemona tuberosa Lour. Conclusion: The activity of the novel plant extracts possibly offers a new approach towards successful chemotherapy of the so far chemo-resistant medullary thyroid carcinoma.*

Medullary thyroid carcinoma (MTC) is a rare calcitonin-producing tumor, derived from the parafollicular C-cells of the thyroid. MTC can be sporadic, familial (FMTC), or part of the inherited cancer syndromes Multiple Endocrine Neoplasia type 2A (MEN2A) and type 2B (MEN2B). (1) MTC is inherited in 25-30% of all cases due to mutation in the *RET*-proto-oncogene, located on chromosome 10 (10q11.2). Presently the only treatment option is surgery, as MTC is known to be relatively insensitive to chemo- or radiation therapy.

Increased expression of the anti-apoptotic protein *Bcl-2* is involved in the development and progression of many tumors (2). *Bcl-2* localizes to cellular membranes, particularly in mitochondria, where it stabilizes the transmembrane potential and reduces membrane permeability. *Bcl-2* seems to contribute to tumor cell survival by enhancing the rate of cell proliferation and by allowing tumor cells to escape destruction by effector cells of the immune system.

Caspase-3 is a key protease that is activated during the early stages of apoptosis and, like other members of the caspase family, is synthesized as an inactive proenzyme that is processed in cells undergoing apoptosis by self-proteolysis and/or cleavage by another protease.

The aim of this study was to investigate new approaches using plant extracts against chemo-resistant medullary thyroid carcinoma.

Materials and Methods

Cell culture. Eight continuous cell lines had been previously established and characterized from biochemically and histologically confirmed medullary thyroid carcinomas: MTC-SK (3), SINJ (4), GRS-IV, GRS-V, BOJO, SHER-1, RARE and GSJO (5). The cells were cultured in Ham's F 12 medium (Biowhittaker, Belgium) containing 10% fetal bovine serum (PAA Laboratories, Exton, PA, USA), at a cell density of 2×10^5 cells/ml and incubated at 37˚C in a 5% CO_2 and 90% humidity incubator. Control cells were normal human skin fibroblasts.

Tested substances. Crude extracts of seven *Aglaia* species, of two *Stemona* species and one semisynthetic substance (Artesunate) were tested. Each substance was screened in concentrations between 0.1 and 135 μg/ ml in order to define the LC_{50}.

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a) Aglaia sp: The genus *Aglaia* (Meliaceae) is characterized by the presence of different types of flavaglines, comprising cyclopenta[b]tetrahydrobenzofurans (roc-aglamides), cyclopenta-[bc]benzopyrans (aglains, aglaforbesins, thapsakins) and benzo [b]oxepines (forbaglines, thapoxepines) (6,7). Together with typical bisamides, flavaglines in particular represent a distinct phytochemistry restricted to the genus *Aglaia* (8). Extracts from seven *Aglaia* species were tested: *A. odorata* Lour, *A. basiphylla* A. Gray, *A. gracilis* A.C. Smith, *A edulis* (Roxb.) Wall, *A. tenuicaulis* Hiern, *A. coriacea* Miq. and *A. elaeagnoidea* (A. Juss) Benth.
b) Stemona sp.: The roots of *Stemona* have long been recommended in Chinese and Japanese traditional medicine for the treatment of respiratory diseases, as well as against enteric helminths and ectoparasites on humans and cattle and insect pests. The biological activity of alkaloids, stilbenoides and tocopherols is gaining particular interest. Rare pyrrolo(1,2-a) azepin-alkaloids, so far verified solely in *Stemonaceae*, are responsible for the insecticidal effects (9, 10). Extracts from *Stemona tuberosa* Lour and *Stemona collinsae* Craib were tested.
c) Artesunate: Artesunate is a semisynthetic derivative of artemisinin, the active compound of the Chinese herb *Artemisia annua* L. It shows remarkable activity against *Plasmodium falciparum* and *P. vivax*; it is highly effective in the treatment of severe malaria and shows antiviral properties. Artesunate is also active against cancer (11-13), but has never been tested against medullary thyroid carcinoma.

Cell counting. MTC cells (2 x 10^5 cells/ ml) were incubated in microplates (96-wells, Sarstedt, Germany) treated with the above extracts for 24, 48 and 72 h. Each sample was analyzed in the CASY-1® Cell Counter & Analyzer TTC (Schärfe System, Germany).

WST-1 cytotoxicity assay. MTC cells (2 x 10^5 cells/ ml) were incubated in microplates (96-well, Sarstedt) treated with the above plant extracts for 24, 48 and 72 h. After the incubation period, 10 μl Cell Proliferation Reagent WST-1 was added per well (Roche Diagnostics, Austria). The formazan produced was quantitated by an ELISA plate reader.

DAPI-staining. MTC cells (2 x 10^5 cells/ ml) were treated with different extracts, fixed in methanol and incubated with DAPI (33258, Hoechst, Germany) for 15 min in the dark, pelleted and resuspended in 25 μl HBSS. The highly condensed chromatin of apoptotic cells was visualized by fluorescence microscopy.

Caspase-3. MTC cells were treated with different extracts, washed in PBSA, fixed and permeabilized using the Cytofix/Cytoperm™ Kit (BD Biosciences, Austria) for 20 min at room temperature, pelleted and washed with Perm/Wash™ Buffer (BD Biosciences). Cells were then stained with PE-conjugated rabbit anti-active caspase-3 mAB using 20 μl/ 1x10^6 cells for 60 min at room temperature in the dark. After the incubation, the cells were washed in Perm/WashTM Buffer twice and analysed by flow cytometry (Partec Pas III, Germany).

Bcl-2. MTC cells were treated with different extracts, washed in PBSA, fixed and permeabilized using the Cytofix/Cytoperm™ Kit (BD Biosciences) for 20 min at room temperature, pelleted and washed with Perm/Wash™ Buffer (BD Biosciences). Cells were then stained with PE-conjugated Hamster anti-human Bcl-2 monoclonal AB (BD Biosciences) using 20 μl/1x10^6 cells for 60 min at room temperature in the dark. After incubation, the cells were washed in Perm/Wash™ Buffer twice and analysed by flow cytometry (Partec Pas III).

Results

The LC_{50} was determined for each substance. Figures 2 and 3 demonstrate the effects of crude extracts of *Stemona tuberosa* Lour on proliferation and caspase-3 activity on the MTC cell line GSJO.

Aglaia edulis (Roxb.) Wall, *A. gracilis* AC Smith, *A. coriacea* Miq., *A. elaeagnoidea* (A. Juss) Benth, *A. odorata* Lour. LC_{50} for each extract was 0.5 μg/ml. The decrease in cell counts was dose-dependent. The accumulation of cells was not influenced. DAPI-staining and *Bcl*-2-analyses showed no apoptotic cells. The highest caspase-3 staining was observed after 48 h and did not exceed 15%.

Aglaia tenuicaulis Hiern. LC_{50} was 30 μg/ml. Antiproliferative effects but no apoptotic signals were observed.

Aglaia basiphylla A. Gray. LC_{50} was 5 μg/ml. Antiproliferative effects but no apoptotic signals were observed.

Stemona tuberosa Lour. LC_{50} was 50 μg/ml. Originally aggregating cells altered their morphology and separated (Figures 1a and b). The numbers and sizes of cells decreased with increasing drug concentration (Figures 2a, b. c). *St. tuberosa* Lour caused apoptotic effects: in contrast to normal cells, the nuclei of apoptotic cells had highly condensed chromatin that was uniformly stained by DAPI. Crescents appeared around the periphery of the nucleus, or the entire nucleus appeared to be one bead, or a group of featureless, bright spherical beads. After 12h, flow cytometric analyses showed no effects on Bcl-2 expression; however, one-third of the treated cells were positive for active caspase-3 staining (Figures 3a, b, c).

Stemona collinsae Craib. LC_{50} was 40 μg/ml. Originally aggregating cells altered their morphology and separated. The numbers and sizes of cells decreased with increasing drug concentration. After 48h, flow cytometric analyses displayed only 14% of treated cells positive for active caspase-3 staining. DAPI-staining showed no apoptotic cells. Artesunate: LC_{50} is 0.04 μml. Antiproliferative effects but no apoptotic signals were observed.

Discussion

A previous study (Strasser *et al.*, [2]) supports the hypothesis that high-level expression of *Bcl-2* confers a chemo-resistant phenotype on tumor cells. We studied the use of plant extracts

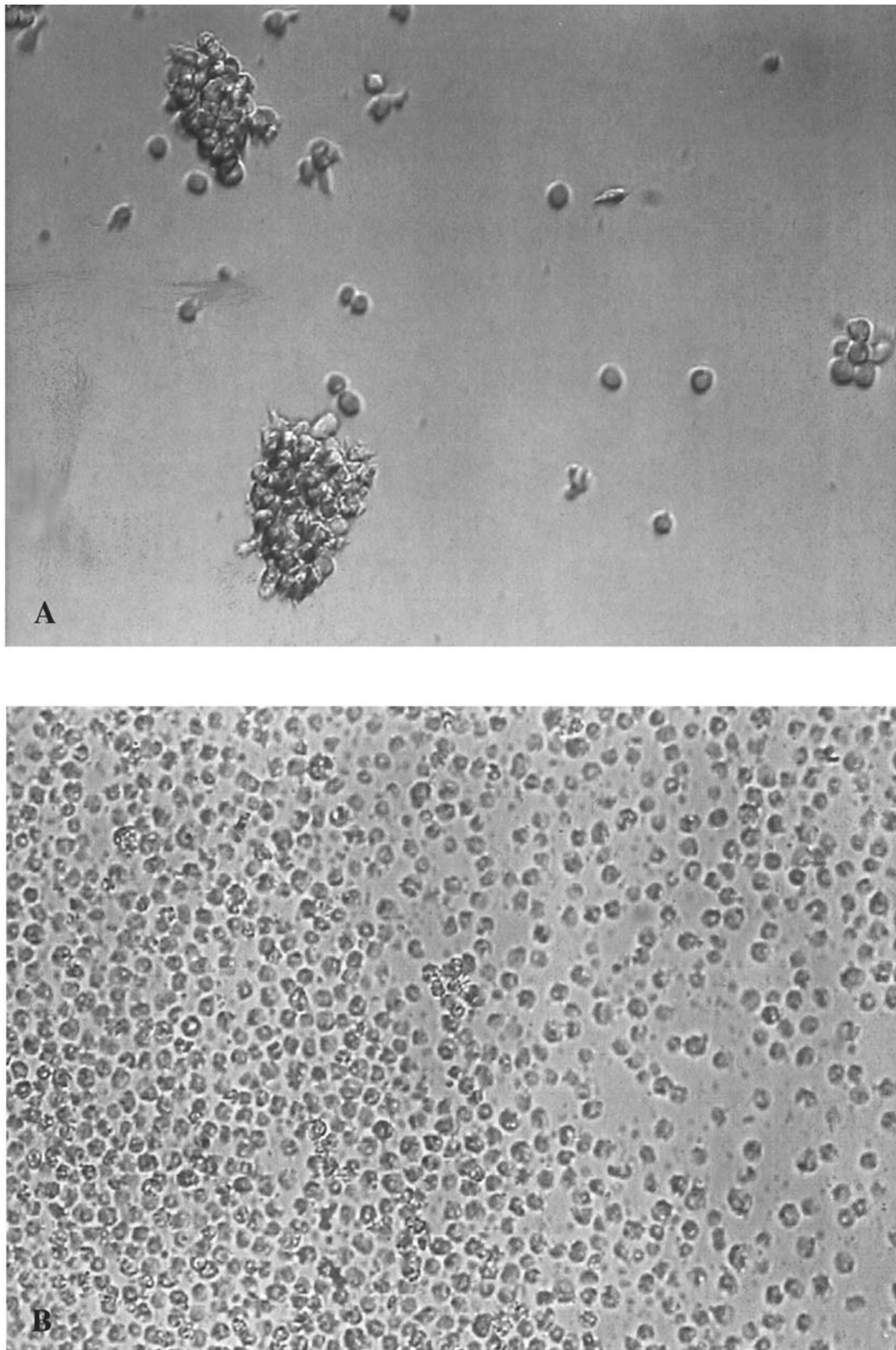
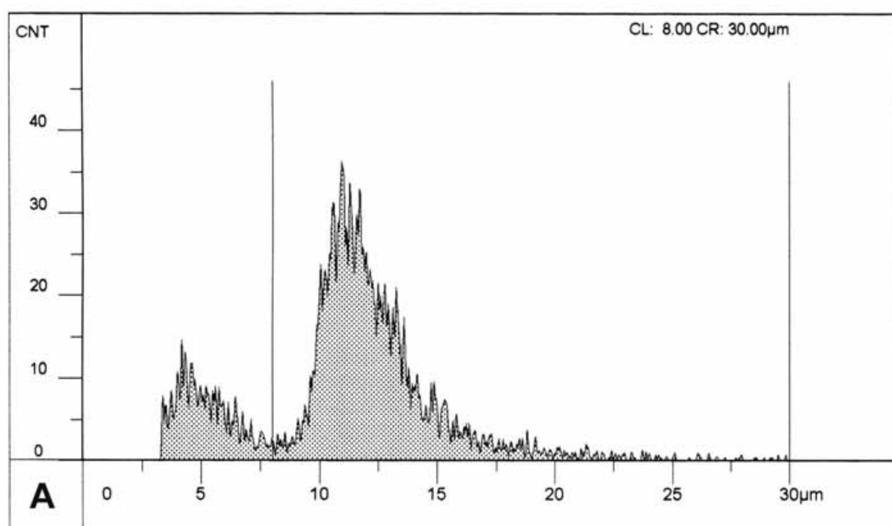
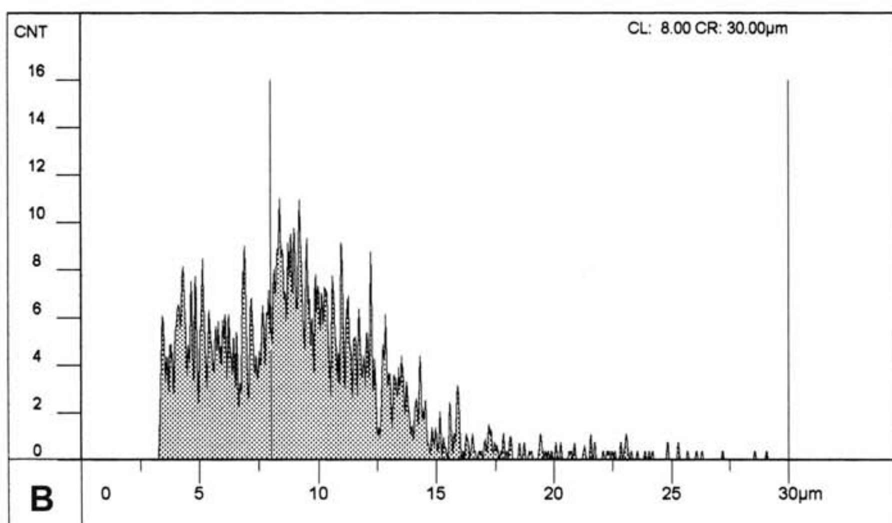


Figure 1. *a) GSJO cell line, growing as a suspension of multicellular aggregates, untreated control. b) Treatment with extracts from S. tuberosa* Lour *(LC_{50}) for 24 h. The multicellular aggregates dissociated.*





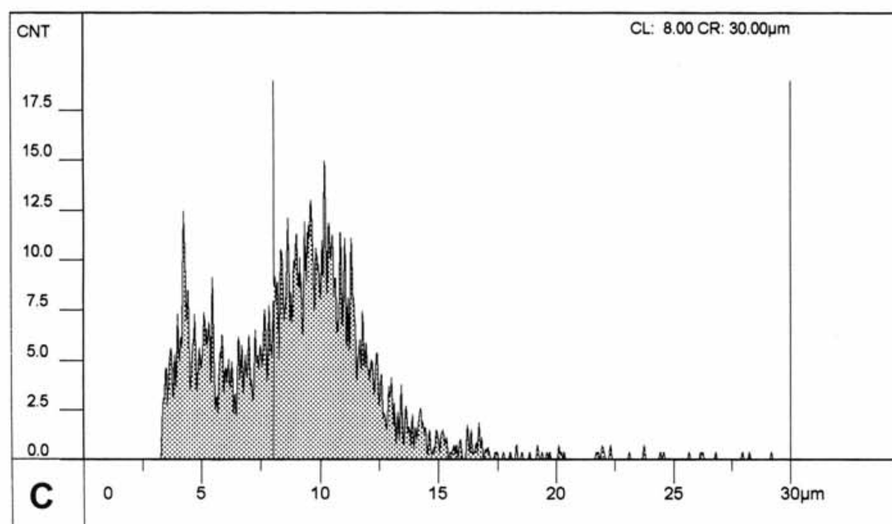


Figure 2. *a) GSJO cell line, cell counts in untreated control. Low percentage of dead cells and cell debris (left peak). b) Cell counts after treatment with S. tuberosa* Lour *extract (LC_{50}) for 24 h. High percentage of dead cells and cell debris (left peak), c) Cell counts after treatment with S. tuberosa* Lour *extract (LC_{50}) for 48 h. High percentage of dead cells and cell debris (left peak)*

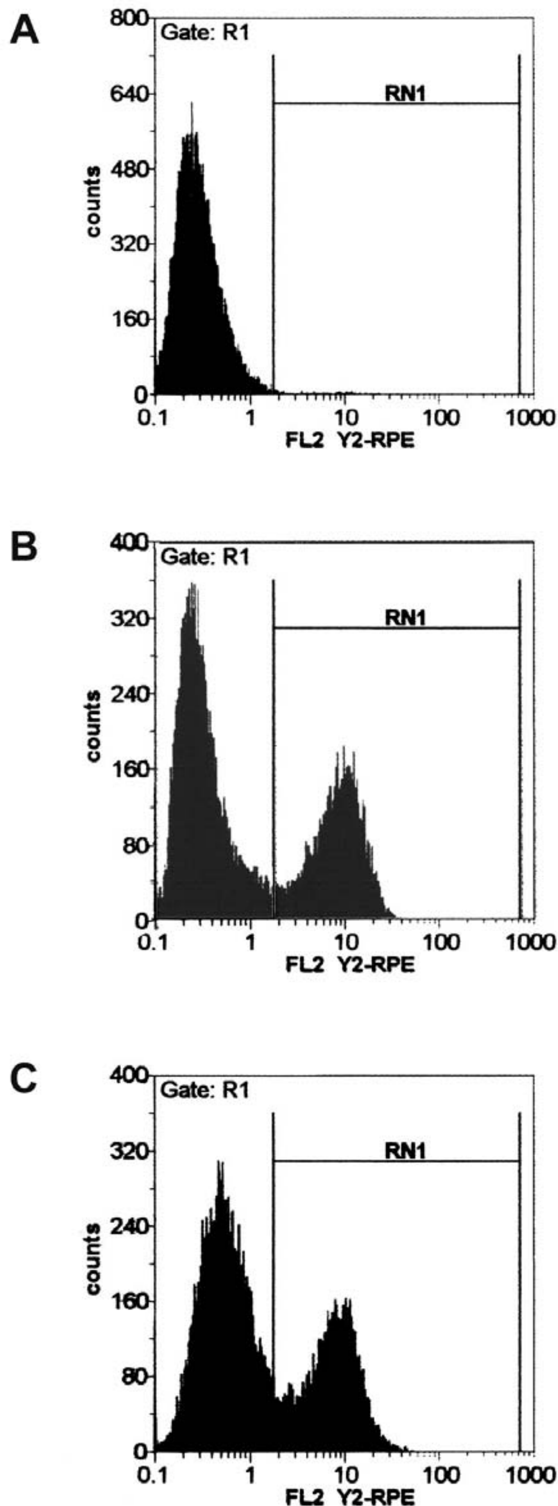


Figure 3. *a) GSJO cell line, flow cytometric analysis of caspase-3 activity, untreated control, 2.42% positive cells. b) Treatment with S. tuberosa* Lour *extract for 24 h, 36.7% positive cells. c) Treatment with S. tuberosa* Lour *extract for 48 h, 37.8% positive cells.*

to inhibit proliferation and to induce apoptosis in medullary thyroid cancer cells. Efferth *et al.* (11 - 13) tested Artesunate on 55 human tumor cell lines (leukemia, melanoma, non-small cell lung cancer, colon cancer, renal cancer, ovarian carcinoma, tumors of the central nervous system, prostate carcinoma and breast cancer). All tumor cell lines were sensitive towards the drug. In our study, the MTC cell lines became necrotic after Artesunate treatment but apoptosis was not found. Extracts of *Aglaia sp.* had a similar effect; cell death could be quickly achieved, but it was not possible to observe an increase in caspase-3 or positive DAPI-staining.

However, extracts from different *Stemonaceae* caused different effects: *Stemona collinsae* Craib had a moderately enhancing effect on apoptosis, while *Stemona tuberosa* Lour enhanced apoptosis considerably. Both extracts from *S. tuberosa* Lour and *S. collinsae* Craib altered the phenotype of the MTC cells from originally aggregating cells towards single-cell suspensions. We assume that the loss of cellular aggregation actually contributes to the effects of the tested substances.

Compared with the effects of the other extracts from *Aglaia sp.* and Artesunate, *Stemona* extracts had the strongest apoptotic effects on MTC cells. *Stemona tuberosa* Lour in particular showed the most promising results in MTC cells. Normal cells were less impaired.

In summary, our findings demonstrate that the proliferation of medullary thyroid carcinoma cells is influenced by different plant extracts, and that *Stemona* does induce apoptosis. *Stemonaceae* had not been tested before on MTC cells, but the activity of the lipophilic plant extracts possibly offers a new approach towards successful chemotherapy of the so far chemo-resistant medullary thyroid carcinoma.

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