Anti-tumor effects of shikonin derivatives on human medullary thyroid carcinoma cells

Carina Hasenoehrl, Gert Schwach, Nassim Ghaffari-Tabrizi-Wizsy, Robert Fuchs, Nadine Kretschmer, Rudolf Bauer and Roswitha Pfragner

1Institute of Pathophysiology and Immunology, Center of Molecular Medicine, Medical University of Graz, Graz, Austria
2SFL Chicken CAM Lab, Institute of Pathophysiology and Immunology, Medical University of Graz, Graz, Austria
3Department of Pharmacognosy, Institute of Pharmaceutical Sciences, University of Graz, Graz, Austria
†(C Hasenoehrl is now at Institute of Experimental and Clinical Pharmacology, Medical University of Graz, Graz, Austria)

Abstract

New treatment options are needed for medullary thyroid carcinoma (MTC), a highly metastasizing neuroendocrine tumor that is resistant to standard radiotherapy and chemotherapy. We show that the following shikonin derivatives inhibit cell proliferation and cell viability of the MTC cell line TT: acetylshikonin, β,β-dimethylacrylshikonin, shikonin and a petroleum ether extract of the roots of Onosma paniculata containing several shikonin derivatives. The unsubstituted shikonin derivative was found to be the most effective compound with an IC₅₀ of 1.1 µM. The cell viability of normal human skin fibroblasts, however, was not affected by the tested substances, indicating that shikonin derivatives might be selectively toxic for cancer cells. We further report that migration and invasion of TT cells were inhibited at non-toxic concentrations. Finally, shikonin was tested in vivo using the chick chorioallantoic membrane assay, where it significantly reduced tumor growth by inhibiting cell proliferation and inducing apoptosis. In summary, our results suggest that shikonin derivatives have the potential for the treatment of medullary thyroid carcinomas.

Introduction

Medullary thyroid carcinomas (MTC) arise from the parafollicular C-cells of the thyroid and account for 5–10% of all thyroid cancers (1, 2). MTCs are calcitonin-producing tumors that occur sporadically in 70–80% of the cases. The remaining 20–30% are hereditary forms that are inherited in an autosomal-dominant pattern, either as part of multiple endocrine neoplasia syndrome type 2A (MEN2A) or 2B (MEN2B), or without any other associated endocrinopathies as familial medullary thyroid carcinoma (FMTC) (3, 4, 5).

MEN2 is characterized by germline mutations of the RET proto-oncogene. Almost all of them are missense mutations leading to increased function of the RET receptor tyrosine kinase. Oncogenic RET mutations of MEN2 are concentrated in a small sequence of the open reading frame and show striking correlations with the phenotype of the MEN2 variant. The most common variant, MEN2A, is mainly caused by mutations in the cysteine-rich portion of the extracellular domain (52% occur in codon 634), and additionally, predisposes to pheochromocytoma and to parathyroid hyperplasia. In MEN2B, the mutation is confined to one cytoplasmic amino acid substitution – Met918Thr. MEN2B has an earlier age of onset than MEN2A and a more aggressive...
course including pheochromocytoma, mucosal neuromas, megacolon and a Marfanoid habitus (3, 6, 7).

MTCs do not respond to conventional therapies like radiation or chemotherapy, and up to 80% of patients present with nodal metastases at the time of diagnosis, so that surgical removal of all neoplastic tissue is the best option. Recurrence, however, is common, frequently with metastases in the bones, lungs, liver and brain. To date, no effective treatment for distant metastases in MTC has been found (1, 8), and the search for new treatment options is thus highly warranted.

Roots of Boraginaceae genera Arnebia, Lithospermum and Onosma, in China also known as Zicao, are rich in shikonin constituents and have been used in traditional Chinese medicine for hundreds of years (9, 10). The medicinal relevance of the active compounds of their extracts was confirmed for the first time in 1978 (11). Since then, various pharmacological effects have been attributed to shikonin derivatives, including acceleration of wound healing, suppression of local acute inflammatory reactions, inhibition of angiogenesis and antimicrobial and cardioprotective activity (9, 12). Lately, numerous studies investigating anti-cancer effects of shikonin have been published. As reviewed by Andújar and coworkers (13), shikonin exerts its anti-tumor effects in a great variety of cancer cell lines through inhibition of cell proliferation and induction of apoptosis. Furthermore, shikonin has been shown to be effective in animal models (14, 15, 16) and in a clinical trial of late-stage lung cancer patients (17).

In the present study, we investigated for the first time the effect of a petroleum ether root extract of Onosma paniculata Bureau & Franchet on a cell line derived from multiple endocrine neoplasia syndrome type 2A (MEN2A). Additionally, we compared its active constituents acetylshikonin and β,β-dimethylacrylshikonin to the unsubstituted shikonin derivative.

**Materials and methods**

**Cell culture**

TT cells, obtained from the European Collection of Authenticated Cell Cultures (ECACC; Porton Down, Salisbury, UK), were cultivated in Ham’s F12 Nutrient Mixture (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS Gold; PAA Laboratories, Pasching, Austria). Cells were passaged at approximately 80% confluence to an initial cell number of 2 × 10^5 cells/mL using trypsin–EDTA (PAA Laboratories).

Normal human skin fibroblasts, HF-SAR (18), were cultured in EMEM (BioWhittaker, Lonza, Verviers, Belgium) supplemented with 2 mM L-glutamine (PAA Laboratories) and 10% FBS. Cells were passaged to an initial cell number of 1 × 10^5 cells/mL using Accutase (PAA Laboratories). All cells were kept in a humidified 5% CO₂ atmosphere at 37°C.

**Shikonin derivatives**

As described previously (19, 20), shikonin derivatives were isolated from Onosma paniculata Bureau & Franchet. Briefly, a petroleum ether extract (EX) was prepared by exhaustive Soxhlet extraction and further fractionated using preparative HPLC. The main components were β,β-dimethylacrylshikonin (DMAS, 38.2%) and acetylshikonin (AS, 24.5%) as identified by NMR spectroscopy. For further comparison of the activity, shikonin (SHK) was purchased from Sigma-Aldrich.

Aliquots of the substances were freshly dissolved in DMSO (Sigma-Aldrich) every second week to ensure consistent bioactivity. Concentrations of DMSO after application of the compounds never exceeded 0.5%, which did not affect cell behavior as controlled by benchmark tests.

**Growth inhibition assay**

Aliquots (2 mL) of TT cells were seeded into 6-well plates (Sarstedt, Wiener Neudorf, Austria) at 2 × 10^5 cells/mL. After allowing the cells to adhere overnight, seven different concentrations of each substance were added. Seventy-two hours later, cells were detached with trypsin–EDTA (500 µL, 3 min). Then, 1.5 mL of FBS-containing medium was added and cells were counted in triplicates using a CASY1 Cell Counter & Analyzer TTC (Schärfe System, Reutlingen, Germany).

All assays were performed with at least three different passage numbers and with medium containing 10% FBS, but no antibiotics. IC₅₀ values were calculated with Microsoft Excel 2010.

**Cell viability assay**

Cell viability of TT cells as well as normal human skin fibroblasts, HF-SAR, was assessed using the Cell Proliferation Reagent WST-1 (Roche Diagnostics). Cells were seeded into 96-well plates in aliquots of 100 µL with a cell density of 1 × 10⁵ cells/mL and 3 × 10⁴ cells/mL, respectively.
After allowing the cells to adhere overnight, the medium was aspirated and replaced with medium supplemented with DMSO (solvent control) or different concentrations of shikonin derivatives as indicated. Samples were tested in 6 replicates after an incubation period of 72h. Results are presented as percentage of solvent-treated control cells.

Cell morphology
Morphological changes occurring after application of shikonin were observed with a Nikon inverted microscope (Eclipse TE 300, Nikon). TT cells (2 mL) were seeded into 6-well plates at a density of 2 × 10⁵ cells/mL. After allowing them to adhere overnight, cells were incubated with shikonin (IC₅₀) and cell morphology was observed after 24 and 48 h. Images were taken with a Nikon 12-bit CCD camera (Nikon).

Monolayer wound-healing assay
TT cells (2 mL) were seeded into 6-well plates at a density of 5 × 10⁵ cells/mL and grown to approximately 90% confluence. The medium was then removed and a scratch was created by scraping the cell monolayer with a p10 pipet tip. Debris was removed by washing the cells with 1 mL PBS, which was then replaced by 2 mL medium supplemented with either DMSO (solvent control) or the corresponding IC₅₀/5 values of the shikonin derivatives. Reference points were created by marking the lid of the plate, and images were acquired with a phase-contrast microscope and a 12-bit CCD camera (Nikon). Cells were incubated at 37°C, 5% CO₂ and observed periodically until the control cells closed the scratch. Experiments were performed with three different passages, and analysis was done using TScratch software (21).

Matrigel invasion assay
Matrigel invasion assays (BD Biosciences) followed the manufacturer’s protocol. Briefly, 1 × 10⁵ TT cells were seeded per well, incubated with either DMSO as solvent control or the corresponding IC₅₀/5 values of the shikonin derivatives. Invaded cells were detected with DAPI staining (Roche Diagnostics) and fluorescence microscopy (Leica DM 4000B; Leica) after 48 h.

Ex ovo chick chorioallantoic membrane assay
Fertilized white leghorn chicken eggs from a local hatchery were incubated at 37.6°C and 70% humidity (J. Hemel Breeding Machines, Germany). The eggshell was cracked on the third day after fertilization and the embryo was decanted to a sterile dish. On day 10, 1 × 10⁶ TT cells were resuspended in 15 μL PBS and 5 μL Matrigel matrix (BD, Biosciences) and grafted in the center of a 5 mm silicon ring on the surface of the chorioallantoic membrane (CAM). Xenografts were treated topically every day, either with 2.2 μM shikonin (2 × IC₅₀) in 10 μL PBS (n = 9) or with 0.02% DMSO in PBS (n = 12) for 3 days. On day 4 after seeding, the xenografts were photographed with a stereo microscope (Olympus SZX16), excised with the surrounding CAM, and then fixed in 4% paraformaldehyde followed by paraffin embedding and cutting of 5-μm sections.

Analysis of proliferation and apoptosis of CAM xenografts
The xenograft sections were processed and stained with an antibody against Ki-67 (clone MIB-1, 1:100; Dako) for the analysis of mitotically active cells. Apoptosis was assessed by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL; Abcam) according to the manufacturer’s instructions. The images were digitally recorded at a magnification of 400× with an Olympus BX53 microscope and an Olympus DP27 camera. Areas from the digitalized color photomicrographs were analyzed. Two examiners independently determined the percentage of positive cells.

Statistical analysis
All data are expressed as means ± standard deviation (s.d.) unless indicated otherwise. The level of significance between two groups was assessed by two-tailed unpaired Student’s t-test using the GraphPad Prism 4.0 statistics software (GraphPad Software). P values <0.05 were considered statistically significant.

Results
Shikonin derivatives exert anti-proliferative effects on TT cells
The effects of acetylshikonin (AS), β,β-dimethylacrylshikonin (DMAS), shikonin (SHK) and a petroleum ether extract of the dried roots of Onosma paniculata (EX) on the cell viability of medullary thyroid
carcinoma cell line TT were examined at 0.1, 1 and 10 µM (for AS, DMAS and SHK) and 0.1, 1 and 10 µg/mL for the extract, respectively. Although the lowest concentration of the pure substances or the extract, respectively, had only a small effect, there was a pronounced reduction in cell viability at 1 µM or 1 µg/mL. 10 µM or 10 µg/mL, respectively, had already reduced cell viability drastically within 24 h (Fig. 1A). To determine the IC₅₀ values of each substance, we incubated TT cells with 0.1–2 µM (for AS, DMAS and SHK) or 0.1–2 µg/mL (EX) for 72 h. A dose-dependent reduction of cell proliferation was seen for all examined compounds (Fig. 1B). Table 1 shows IC₅₀ values as mean ± S.D. of at least three independent experiments. To allow for comparison of potency of the pure substances with the unfractinated extract, IC₅₀ concentrations of acetylshikonin, β,β-dimethylacrylshikonin and shikonin were converted to µg/mL according to their molecular weights (Fig. 1C). The application of the extract, which is a mixture of several shikonin derivatives, did not produce lower IC₅₀ values than application of the pure compounds. In fact, shikonin and β,β-dimethylacrylshikonin exhibited the lowest IC₅₀ values on TT cells.

Figure 1
Shikonin derivatives inhibit proliferation of TT cells in a dose-dependent manner but do not reduce cell viability in normal human skin fibroblasts. (A) TT cells were incubated with 0.1, 1 and 10 µM acetylshikonin (AS), β,β-dimethylacrylshikonin (DMAS) or shikonin (SHK) and 0.1, 1 and 10 µg/mL petroleum ether extract of Onosma paniculata roots (EX). Cell viability was reduced at 1 µM concentrations of acetylshikonin, β,β-dimethylacrylshikonin and shikonin and 1 µg/mL of the extract. Concentrations of 10 µM and 10 µg/mL had deleterious effects on cell viability. Experiments were performed three times in sextuplicates. (B) Cell proliferation of TT cells as assessed by cell counting was dose dependently inhibited by acetylshikonin, β,β-dimethylacrylshikonin, shikonin and the extract. Cell numbers are presented as percentage of control solvent-treated cells, n = 3–4. (C) Conversion of determined IC₅₀ values (Table 1) from µM to µg/mL allows comparison of potency between pure substances and the unfractinated extract. Shikonin and β,β-dimethylacrylshikonin were found to reduce cell proliferation at lower concentrations than the extract. (D) Cell viability of HF-SAR skin fibroblasts, on the contrary, was not significantly reduced when cells were incubated with the IC₅₀ concentrations of shikonin derivatives determined for TT cells. Results were normalized to control solvent-treated cells, n = 3.
Shikonin derivatives do not impair cell viability of skin fibroblasts

Normal HF-SAR human skin fibroblasts were incubated with shikonin derivatives at the IC₅₀ concentrations determined for TT cells. Treatment with acetylshikonin did not affect the metabolic activity of HF-SAR cells, whereas treatment with β,β-dimethylacrylshikonin, shikonin and the extract caused a slight albeit statistically non-significant reduction in cell viability (Fig. 1D).

Shikonin derivatives induce morphological changes in TT cells

The reduction of cell viability and proliferation caused by treatment with shikonin derivatives was accompanied by morphological changes in TT cells. Incubation with the corresponding IC₅₀ concentrations caused detachment and rounding of cells. Figure 2 shows images of TT cells treated with DMSO as solvent control and representative images after incubation with the IC₅₀ concentration of shikonin derivatives.

Cancer cell migration and invasion are inhibited at non-toxic concentrations

Migration of TT cells was assessed with wound-healing assays (Fig. 3A, B, C, D, E, F, G, H, I and J). Control solvent-treated TT cells were able to close the created gap within nine days, whereas treatment with shikonin derivatives at non-toxic concentrations (IC₅₀/5) inhibited cell migration. Wound closure was significantly decreased (P<0.05) after treatment with 0.2 µM shikonin, and very significantly (P<0.01) after application of 0.4 µM acetylshikonin, 0.3 µM β,β-dimethylacrylshikonin or 0.15 µg/mL petroleum ether extract (Fig. 3K).

Additionally, the invasive behavior of TT cells was studied using Matrigel invasion assays. Invasion was significantly inhibited after application of 0.1 µM shikonin (IC₅₀/10, P<0.05) and 0.2 µM shikonin (IC₅₀/5, P<0.01) (Fig. 3L).

Shikonin inhibits cell proliferation and induces apoptosis in CAM xenografts

As the investigated shikonin derivatives showed promising anti-tumor effects in vitro, we next explored...
Shikonin induces apoptosis in MTC cells

The effect of shikonin in a tumor xenograft of TT cells on chorioallantoic membranes (Fig. 4A and B). In this model of in vivo tumorigenesis, treatment with 2.2 µM shikonin (2 × IC50) gave cancer cells the appearance of an apoptotic phenotype without affecting the surrounding CAM (Fig. 4C and D). Immunohistochemical staining of cross-sections further revealed a significant (P < 0.05) decrease in proliferating cancer cells after shikonin treatment, as observed by Ki67 staining (Fig. 4E, F and I). Parallel sections were analyzed for TUNEL staining, which is an indicator of apoptosis. Treatment with shikonin highly significantly increased (P < 0.001) the number of TUNEL-positive TT cells compared to solvent control treatment (Fig. 4G, H and J), indicating that shikonin exerts its anti-tumorigenic effects through inhibition of cell proliferation as well as induction of apoptosis in cancer cells.

Discussion

Although medullary thyroid carcinoma is a rare hormone-producing tumor, it accounts for 13.4% of deaths attributable to thyroid cancers (22). As MTC does not respond to conventional therapy, novel treatment options...
are needed (1, 2, 8, 23) and compounds targeting the RET kinase are currently under investigation (24). Although promising results were achieved with other cancers, the response rates for MTC were low (25).

We investigated the effects of various shikonin derivatives, active compounds of the dried roots of Boraginaceae that are used as herbal anti-cancer medicine in China, on TT cells derived from a patient diagnosed with MEN2A (26). Shikonins are known to possess several pharmacological effects including antibacterial, wound-healing, anti-inflammatory and anti-cancer properties (9, 10, 11, 12, 13). The anti-cancer properties of shikonin include its ability to induce apoptosis and inhibit cell proliferation (11, 13). In addition, shikonin has been shown to inhibit the proliferation of cancer cells in vitro and in vivo (9, 12, 14).

**Figure 4**
Shikonin reduces cell proliferation and induces apoptosis in chicken chorioallantoic membrane xenografts (A, B, C, D, E, F, G and H). Representative images show TT xenografts treated with solvent control (control, left row, n = 12) or with 2.2 µM shikonin (SHK, right row, n = 9). (A and B) The CAM assay was used for *in vitro* testing of shikonin treatment of TT cells. Xenografts were photographed through a stereo microscope. Scale bars, 1 mm. (C and D) Hematoxylin–eosin (H&E) staining of cross-sections of TT grafts in the CAM. Arrows in (D) indicate apoptotic cells. Scale bars, 100 µm. (E, F, G and H) Immunohistochemical staining of TT cells grown on CAM using an antibody directed against the proliferation marker Ki67. Parallel samples were subjected to TUNEL staining. Scale bars, 20 µm. (I and J) Quantification of Ki67- and TUNEL-stained sections of TT grafts. The percentage of Ki67-positive cells was significantly (*P* < 0.05) reduced after shikonin treatment, whereas the percentage of TUNEL-positive cells was highly significantly increased (***P*** < 0.001). Data are shown as mean ± s.e.m.
effects have been shown to be linked to fundamental cellular processes such as cell cycle progression, apoptosis (19, 20) and mitochondrial function (27, 28). Shikonins further affect main anti-apoptotic and pro-proliferative pathways such as MAPK and AKT signaling cascades (29, 30), but the effects on MTC cells with respect to migration and invasion as well as in vitro tumor growth have not yet been investigated. IC values on TT cells were found to be in the micromolar range with shikonin exhibiting the lowest IC of 1.1 µM. The potencies of the pure substances acetylskokin, β,β-dimethylacrylskokin and skokin were compared with the unfraccionated petroleum ether extract by converting the IC values from µM to µg/mL. Interestingly, application of the extract, which contains several skokin derivatives (19), did not show better anti-tumor efficiency than single pure compounds. In fact, the IC of the extract (i.e. 0.56 ± 0.25 µg/mL) was found to be between the IC values determined for acetylskokin (i.e. 0.69 ± 0.15 µg/mL) and β,β-dimethylacrylskokin (i.e. 0.44 ± 0.10 µg/mL) as shown in Fig. 1C and Table 1. We so conclude that the main compounds of the root extract, i.e. acetylskokin and β,β-dimethylacrylskokin, are indeed the compounds responsible for the anti-tumor effects of the root extract but that there is no synergistic effect of the skokin derivatives. Overall, skokin derivatives seem to be highly efficient in the inhibition of medullary thyroid carcinoma cell proliferation as compared to e.g. papillary thyroid cancer cell lines where IC values of 4.20–11.50 µM have been reported (16).

In glioblastoma cells U87MG, various skokin derivatives have been reported to have cytotoxic properties with IC values ranging from 3.61 µM to 51.97 µM (30). In combination with the tyrosine kinase inhibitor erlotinib, however, synergistic effects of e.g. acetylskokin and β,β-dimethylacrylskokin have been observed that were attributed to an inhibition of receptor tyrosine kinase phosphorylation and reduced phosphorylation of downstream signaling molecules (30). Whether synergy of skokin derivatives with tyrosine kinase inhibitors could also be achieved in medullary thyroid carcinoma is thus of high interest for future research.

As the desired outcome in preclinical anti-cancer drug research is the induction of apoptosis in cancer cells but not in normal cells, we treated normal human skin fibroblasts with the skokin derivatives at concentrations determined to reduce cell viability of cancer cells by 50%. We observed that these concentrations did not, or only slightly, reduce cell viability of HF-SAR cells, indicating selective cytotoxicity of skokin and its derivatives on tumor cells.

Besides inducing apoptosis and inhibiting cell growth, the ability of anti-cancer drugs to inhibit cell invasion and metastasis is considered fundamental. Accordingly, we also examined the potential of skokin derivatives to inhibit cell migration and invasion in vitro. We found that sub-lethal concentrations (IC/5 and IC/10) sufficed to inhibit cell migration and invasion of TT cells. As shown in Fig. 3K, we observed a comparative efficiency of all tested skokin derivatives as the corresponding IC/5 concentration reduced wound closure by 53% (acetylskokin), 55% (β,β-dimethylacrylskokin), 59% (skokin) and 59% (extract), respectively.

Finally, we assessed the effects of skokin treatment on TT xenografts. Although the application of skokin did not affect the surrounding cells of the chicken chorioallantoic membrane, it caused a reduction of proliferating cancer cells. Concomitantly, there was a strong increase in TUNEL-stained cells after treatment with skokin, indicating the induction of apoptosis in tumor cells. To the best of our knowledge, this is the first report of skokin derivatives inducing apoptosis in an in vivo model of medullary thyroid carcinogenesis.

Conclusion

Shikonin and its derivatives display a wide spectrum of activity. As recently summarized by Andújar and coworkers (13), shikonin induces apoptosis in a variety of cancer cell lines but not in normal cell lines. In this study, we show that shikonin and its derivatives induce apoptosis and also inhibit cell migration and invasion in a cell line derived from medullary thyroid carcinoma, a tumor that is resistant to cytotoxic chemotherapy. Shikonin has, additionally, been shown to prevent cells from acquiring drug resistance (31), and thus, the targeted application of Onosma paniculata extracts and their active constituents could offer a new option in the treatment of MTC.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
Shikonin induces apoptosis in MTC cells

Funding
This work was supported by the Austrian Science Fund (FWF, P27505), the Jubilee Fund of the Austrian National Bank Project #14394 and the Franz Lanyar Project #380.

Author contribution statement
R P, G S and N G T W designed the study. N K and R B provided the O. paniculata extract and extracted the pure shikonin compounds. C H and N G T W performed the experiments. All authors participated in the writing of the manuscript.

Acknowledgements
The authors thank Eugenia Lamont (Medical Editor and Translator, Medical University of Graz, Graz, Austria) for critically reading the manuscript. We gratefully thank SFL Technologies (Stallhofen, Austria) for providing us with the Olympus SZX16 stereomicroscope.

References
Research

C Hasenoehrl et al. Shikonin induces apoptosis in MTC cells


