ANTI-TUMOR EFFECTS OF SHIKONIN DERIVATIVES ON MEDULLARY THYROID CARCINOMA CELL LINES

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ZUSAMMENFASSUNG

Das medulläre Schilddrüsenkarzinom (MTC) ist ein Calcitonin produzierender neuroendokriner Tumor der parafollikulären Zellen der Schilddrüse, welche ob ihrer Funktion auch C-Zellen genannt werden. Die Chance auf Heilung besteht derzeit nur dann, wenn das gesamte neoplastische Gewebe mittels radikaler Resektion der Schilddrüse entfernt wird. Allerdings sind bei mehr als einem Viertel der Patienten zum Zeitpunkt der Erstdiagnose bereits Fernmetastasen vorhanden, die außerdem resistent gegenüber herkömmlicher Strahlen- und Chemotherapie sind. Die Etablierung von neuen Methoden zur Behandlung von medullärem Schilddrüsenkarzinom ist daher von äußerster Bedeutung.

Shikoninderivate finden schon seit Jahrtausenden Anwendung in der traditionellen chinesischen Medizin (TCM). In letzter Zeit wurde vermehrt berichtet, dass diese auch Antitumorwirkung zeigen und in verschiedensten Krebszelllinien Apoptose auslösen. Ziel dieser Arbeit war es, den Effekt der Reinsubstanzen Acetylshikonin, Dimethylacrylshikonin, Shikonin und des Petroleumetherextrakts der Wurzeln von *Onosma paniculata* erstmals auf medullären Schilddrüsenkarzinomzelllinien zu untersuchen.

Die Ergebnisse zeigen einen zeit- und dosisabhängigen inhibitorischen Effekt aller getesteten Substanzen auf Zellproliferation und mitochondriale Aktivität der MTC-Zelllinien MTC-SK, SHER-I und TT. Die IC_{50}-Werte der jeweiligen Substanzen waren ≤ 0.5µM für die Suspensionszelllinien und betrugen 1-2µM für die adhärent wachsenden Zellen. Im Gegensatz dazu hatten diese Konzentrationen keinen hemmenden Einfluss auf normale humane Hautfibroblasten, die als Kontrolle dienten. Weiters konnten in den Tumorzelllinien morphologische Veränderungen wahrgenommen werden; Suspensionszellen verloren die Fähigkeit multizelluläre Aggregate zu bilden während die adhärenten Zellen nach Behandlung mit den IC_{50}-Konzentrationen rund anstatt vieleckig waren. Veränderungen der Zellkerne, welche auf Induktion der Apoptose hindeuten, z.B. Kondensierung des Chromatins und Bildung von apoptotischen Zellfragmenten, konnten ebenfalls beobachtet

werden. Die Aktivierung der Apoptose konnte auch durch Messung von erhöhter Caspase 3/7-Aktivität festgestellt werden. Bestätigt wurden diese Ergebnisse mittels durchflusszytometrischer Untersuchung von Annexin V-Bindung und Zellzyklusanalyse. Die Behandlung mit Acetylshikonin oder Shikonin hatte jeweils zur Folge, dass Zellen in der G0/G1-Phase stehen blieben. Die gemessene Depolarisierung der Mitochondrienmembran weist außerdem ebenfalls auf apoptotische Vorgänge hin.

Die Migration und Invasion der Tumorzellen wurde bereits durch sub-toxische Konzentrationen von Shikonin inhibiert. Die Hypothese, dass dies durch verringerte Expression von MMP9 und/oder Metadherin bedingt sei, wurde allerdings widerlegt.

Alles in allem deuten die gefundenen Resultate darauf hin, dass die gezielte Anwendung von *O. paniculata* Extrakten beziehungsweise deren aktiven Komponenten durchaus eine neue Möglichkeit zur Behandlung des medullären Schilddrüsenkarzinoms darstellen könnte.

ABSTRACT

Medullary thyroid carcinoma (MTC) is a calcitonin-producing neuroendocrine tumor of the parafollicular cells, or C-cells, of the thyroid. Presently, the only potentially curative treatment is the complete removal of all neoplastic tissue. However, more than a quarter of the patients presents with distant metastases at the time of initial diagnosis. Moreover, MTC show a poor response to standard radio- and chemotherapy. New treatment options are thus highly needed.

Shikonin and its derivatives have been used in traditional Chinese medicine against various diseases. Recently it has been shown that they also exhibit anti-tumor effects and induce apoptosis in various cancer cell lines. This study aimed to investigate the effects of acetylshikonin, dimethylacrylshikonin, shikonin and a petroleum ether extract of the roots of *Onosma paniculata* on MTC cell lines.

All examined shikonin derivatives showed time- and dose-dependent inhibitory effects on cell proliferation and viability of MTC cell lines MTC-SK, SHER-I and TT. IC_{50} values were found to be ≤ 0.5µM for suspension cells and 1-2µM for adherently growing cells. On the contrary, these concentrations did not reduce cell viability of normal human skin fibroblasts, HF-SAR. Morphological changes were observed in MTC cell lines when the IC_{50} concentration of the corresponding shikonin derivative was applied; suspension cells lost the ability to form multicellular aggregates and adherent cells showed a round rather than polygonal shape and detached from the culture flasks. Nucleic alterations conform to the induction of apoptosis (i.e. chromatin condensation and formation of apoptotic bodies) were observed and the activation of apoptotic pathways was confirmed by enhanced activity of effector caspases 3/7 within 6 hours after treatment. These findings were also confirmed by Annexin V binding measurements and cell cycle analysis through flow cytometry. Application of shikonin and acetylshikonin induced a cell cycle arrest in G0/G1 and resulted in the formation of a sub-G0 peak. Breakdown of the

mitochondrial transmembrane potential also indicated the activation of apoptotic pathways.

Cell migration and invasion were reduced at sub-toxic concentrations as demonstrated by monolayer wound healing assay and Matrigel assay, respectively. The hypothesis that the reason therefor is the downregulation of expression of MMP9 and/or metadherin, both proteins involved in cell migration, was not proven true.

Taken together, our findings suggest that the targeted application of *O. paniculata* extracts and its active constituents could offer a new possibility for the treatment of medullary thyroid carcinoma.

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Last but not least, I would like to thank my friends for supporting me during my studies and while writing this thesis. Special thanks to Ceci, for assisting me with all the images and the layout, for adding all the commas I forgot, and for being just the best companion ever.

Hoping to have chosen a better approach than above depicted, I dedicate this thesis to all patients suffering from cancer. May they never lose hope.

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LIST OF ABBREVIATIONS

$[Ca^{2+}]_i$	intracellular calcium concentration
5-HT	5-hydroxytryptamine
7-AAD	7-amino-actinomycin D
AD	Anno Domini
ADP	adenosine diphosphate
ANT	adenine nucleotide translocator
Apaf1	apoptotic protease activating factor type 1
array-CGH	comparative genomic hybridization array
AS	acetylshikonin
Asp	asparagine
ATP	adenosine triphosphate
BC	Before Christ
Bcl-2	protein: B-cell lymphoma 2
c-FLIP	cellular FADD-like ICE inhibitory protein
CAD	caspase-activated DNase
CARD	caspase activation and recruitment domain
CASP	caspase
CCR5	C-C chemokine receptor type 5
CDK4	cyclin-dependent kinase type 4
cDNA	complementary DNA
CGRP	calcitonin gene-related peptide
Co	control
CO_2	carbon dioxide
crmA	cytokine response modifier A
dADP	deoxyadenosine diphosphate
DAPI	4',6-diamidine-2'-phenylindole dihydrochloride
dATP	deoxyadenosine triphosphate
DD	death domain
DED	death effector domain
DIABLO	direct IAP-binding protein with low pI
DISC	death-inducing signaling complex

DMAS	β,β-dimethylacrylshikonin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DR	death receptor
ECACC	European Collection of Cell Cultures
EDTA	ethylenediaminetetraacetic acid
EMEM	Eagle's minimal essential medium
ERK	extracellular signal-regulated kinase
EtOH	ethanol
EX	petroleum ether extract
FACS	fluorescence activated cell sorter
FADD	Fas-associating protein with death domain
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FMTC	familial medullary thyroid carcinoma
G0/G1/G2	gap 0/1/2 phase in the cell cycle
GRP	gastrin releasing peptide
GTPase	guanosine triphosphate binding protein
H_2O	aqua bidest.
HBSS	Hanks' buffered salt solution
HPLC	high pressure liquid chromatography
HPRT1	hypoxanthine-guanine phosphoribosyltransferase type 1
IAP	inhibitor-of-apoptosis
IC_{50}	inhibitory concentration of the half-maximal effect
JC-1	5,5′,6,6′-tetrachloro1,1′,3,3′-tetramethylbenzimidazolyl-carbocyanine iodide
JNK	c-Jun N-terminal kinase
M	mitosis
Mcl-1	protein: myeloid cell leukemia 1
MEN 2A	multiple endocrine neoplasia syndrome type 2A
MEN 2B	multiple endocrine neoplasia syndrome type 2B
M-FISH	multiplex fluorescence in situ hybridization
$MgCl_2$	magnesium chloride

MMP9	matrix metalloprotease type 9
mRNA	messenger RNA
MTC	medullary thyroid carcinoma
MTDH	metadherin
NADH	reduced nicotinamide adenine dinucleotide
NET	neuroendocrine tumor
NF-κB	nuclear factor "kappa-light-chain-enhancer" of activate B-cells
NMR	nuclear magnetic resonance
OD	optical density
PAK2	p21 activated kinase type 2
PBSA	Dulbecco's phosphate buffered saline lacking Ca^{2+} and Mg^{2+}
PCR	polymerase chain reaction
PE	phycoerythrin
PET	polyethylene terephthalate
PS	phosphatidylserine
qPCR	quantitative PCR
RAS	rat sarcoma proto-oncogene
RET	rearranged after transfection proto-oncogene
RNA	ribonucleic acid
ROS	reactive oxygen species
SD	standard deviation
Shik	shikonin
Smac	second mitochondria-derived activator of caspases
SRIF	somatotropin release inhibiting factor
TAE	Tris base, acetic acid and EDTA
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRADD	TNF-receptor associated death domain
TRAIL	tumor necrosis factor related apoptosis inducing ligand
UV	ultraviolet
VDAC	voltage-dependent anion channel
VEGFR	vascular endothelial growth factor receptor
WST-1	water-soluble tetrazolium salt 1

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1. INTRODUCTION

1.1 General introduction

Neuroendocrine tumors (NET) of the thyroid are rare calcitonin-producing tumors arising from the parafollicular cells, or C-cells. In 75% of the cases they occur sporadically. The remaining 25% are hereditary forms that are inherited in an autosomal-dominant pattern, either as familial medullary thyroid carcinoma (FMTC) without other associated endocrinopathies or as part of multiple endocrine neoplasia syndrome type 2A (MEN 2A) or 2B (MEN 2B) [1].

Presently, the only potentially curative treatment is the surgical removal of the thyroid. However, at least a quarter of the patients has distant metastases at the time of initial diagnosis. Additionally, medullary thyroid carcinoma (MTC) shows a poor response to chemo- and radiotherapy [2]. An intensive search for new alternative treatment strategies is thus warranted.

The first step in the search for new therapeutical options is the establishment of suitable models. Establishing continuous human MTC cell lines representing different tumor stages, growth dynamics, ages of patients, and sporadic or hereditary forms leads to *in vitro* models that can serve as tools for developing new anticancer therapies [1].

For almost two decades, the investigation of new therapeutic strategies was limited by the availability of only one MTC cell line, TT [3]. Establishing continuous cell lines from solid tumors, especially from a medullary thyroid carcinoma, is problematic since primary cultures have a low survival rate [1].

R. Pfragner, however, developed a protocol that led to the establishment of nine MTC cell lines in her lab so far. Accordingly, one part of this thesis was to set up a primary culture from a MTC and establish a long-term culture.

The second part of this work concerned the investigation of the effect of bioactive agents of *Onosma paniculata*, namely shikonin derivatives, on established MTC cell lines. Ideally, substances investigated for a possible application in cancer treatment trigger antitumor effects and induce apoptosis specifically in tumor cells but not in normal cells. The obtained results contribute to a foundation further research could be based on, eventually leading to the application of the gained knowledge in the treatment of neuroendocrine tumors.

1.2 Medullary thyroid carcinoma

Neuroendocrine tumors (NET) are neoplasms arising from neuroendocrine cells, which form the interface of the hormonal and the nervous system. This integration is facilitated by the release of hormones upon a neuronal input. Neuroendocrine cells are scattered throughout the body and give rise to a variety of tumors. Although NETs mostly occur in the intestine, especially the duodenum and colon, they are also found in the stomach, lung or thyroid [4].

Neuroendocrine tumors originating from parafollicular calcitonin-producing cells of the thyroid (Fig. 1) are termed medullary thyroid carcinoma. Parafollicular cells are located adjacent to thyroid follicles, primarily in the posterior upper third of the lateral lobes, and their principal function is the production and secretion of calcitonin (Fig. 2), which is a peptide hormone that decreases calcium levels in the blood [5].

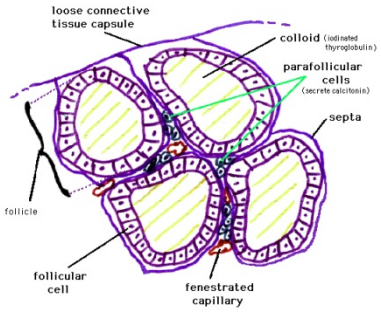


Fig. 1 – PARAFOLLICULAR C-CELLS

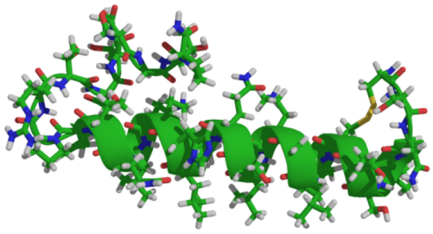


Fig. 2 - CALCITONIN

Medullary thyroid carcinomas comprise 5-10% of all thyroid malignancies with an annual incidence of approximately 0.2 per 100.000 inhabitants [6]. MTC mainly – in 70-80% of the cases – occurs sporadic. The remaining 20-30% are hereditary forms that are inherited in an autosomal-dominant way, either as part of multiple endocrine neoplasia syndrome type 2A (MEN 2A) or 2B (MEN 2B), or without any associated endocrinopathies as familial MTC (FMTC) [2].

More than 95% of MEN2 families show a germline mutation of the rearranged during transfection (*RET*) protooncogene. *RET* is located near the centromere of chromosome 10 and encodes a transmembrane receptor tyrosine kinase of approximately 1.100 amino acids. Mutations of *RET* detected in MEN2 families are almost exclusively missense mutations causing a gain of

function. They are concentrated in a small fraction of the open reading frame encoding the cysteine-rich portion of the extracellular domain; codon 634 has 52% (accountable for MEN 2A and FMTC) and codon 918 has 17% (all as MEN 2B). Oncogenic *RET* mutations thus show striking correlations with the phenotype. The most common phenotypic variant, MEN 2A, occurs as a combination of MTC with associated endocrinopathies like pheochromocytoma (NET of the adrenal gland) and hyperparathyroidism. FMTC show the same mutations as MEN 2A, but since the phenotype does not display any other endocrinopathies, it can be considered as the mild end of the MEN 2A clinical spectrum. In MEN 2B, almost all *RET* mutations are confined to one amino acid – Met918Thr. This variant emerges at a much earlier age and shows a more aggressive course including pheochromocytoma, mucosal neuromas, megacolon and a marfanoid habitus [7].

Spontaneously arising MTC cases, so-called sporadic cases, are caused by somatic *RET* mutations in 50% of the occurrences. No other genes have been consistently implicated in sporadic MTC tumorigenesis yet. [2] Recently, however, RAS has been proposed to act as an alternative driver since mutations in the RAS family of small GTPases genes have been identified in *RET*-negative MTC cases [8].

Patients with MTC usually present with a palpable thyroid nodule and symptoms like dysphagia, hoarseness, shortage of breath, coughing and diarrhea. Since conventional radiation and chemotherapeutic treatment have demonstrated no significant effect on the reduction of tumor mass, treatment entirely relies on radical resection of all neoplastic tissue. Moreover, up to 80% of patients present with nodal metastases, too. Therefore, the operative procedure recommended is a total thyroidectomy combined with removal of the central compartment of lymph nodes and functional neck dissection. However, recurrence is common and frequently metastases in bones, lungs, liver and brain are developed. To date, no effective treatment for distant metastasis in MTC has been found [9].

The main characteristic marker of MTC is a detectable serum calcitonin level. Patients with undetectable calcitonin levels after initial surgery are considered cured whereas recurring disease might result in concentrations

greater than 500 ng/l. Serum calcitonin levels are related to tumor mass and therefore considered as a sensitive disease marker [10].

Parafollicular cells of the thyroid are also known to produce CGRP (calcitonin gene-related peptide), GRP (gastrin releasing peptide), SRIF (somatostatin) and 5-HT (serotonin). Electron microscopy revealed endocrine granules in cells of the original tumor tissue. Subsequently, these characteristic features are also preserved in cultured cell lines as shown by immunohistochemistry [11], [12].

1.3 Establishing cell lines from human neuroendocrine tumors

Human tumor samples obtained from surgery can be used to define their pathology, gene expression and metabolism. However, such pathological samples are complex, heterogeneous and not amenable for experimentation. Therefore, the establishment of appropriate models is required. Although they are not exactly representative of the *in vivo* cell population of cancer, human cancer cell lines are the closest equivalent to tumor initiating cells. Among other things, they allow for testing of hypotheses about the origin and pathogenesis of tumors, the detailed investigation of pathophysiological pathways, the definition of potential diagnostic markers and screening and testing of chemical compounds as potential drugs [13].

Cancer cell lines may show differences in their genetics, epigenetics and gene expression when compared to *in vivo* tumors. Nevertheless, when regarded as a whole, they represent many of the general properties of cancer tissue [13]. On that account, developing cell lines from different tumor stages, different ages of patients at surgery and with different characteristics is crucial.

Samples derived from human neuroendocrine tumors are generally small and show poor initial proliferation *in vitro*. A small proportion of primary cultures, nonetheless, gives rise to continuous cell lines, i.e. continuous growth for > 100 cell divisions or > 1 year [14]. So far, the following human medullary thyroid carcinoma cell lines have been established in our lab (Table 1).

Table 1 – MTC cell lines established in R. Pfragner's lab

Cell line	Tumor stage at time of surgery	Sex / age	Hereditary or sporadic	Primary tumor/ metastasis
BOJO	pT4N2M1	Male / 69	sporadic	Lymphnode-metastasis
GRS-IV	pT4N1M1	Male / 55	sporadic	Lymphnode-metastasis
GRS-V	pT4N1M1	Male / 55	sporadic	Lymphnode-metastasis
HEVE-II	pTxN1bMX	Female / 45	sporadic	Lymphnode-metastasis
MTC-SK	pT4N1MX	Female / 51	sporadic	Lymphnode-metastasis
OEE-III	pT2aN1bM1	Female / 53	sporadic with M918T *RET*-mutation	Lymphnode-metastasis
RARE	pT4N1MX	Female / 53	sporadic	Lymphnode-metastasis
SHER-I	pT4N1MX	Female / 72	sporadic	Primary tumor
SINJ	pT2N1M0	Male / 28	sporadic	Lymphnode-metastasis

1.4 Investigated cell lines: MTC-SK, SHER-I, TT and HF-SAR

Cell line MTC-SK was derived from a lymph node metastasis of a sporadic MTC in a 51 year old female patient. The *in vitro* properties correspond to the results obtained from the original tumor tissue, i.e. production of calcitonin, CGRP and GRP. Cytogenetic studies revealed terminal chromosomal rearrangements concerning chromosome 11p, which is the locus of the calcitonin and calcitonin gene-related peptide genes [12].

SHER-I cells were established from the primary tumor of sporadic MTC in a 72 year old female. Immunocytochemical characterization confirmed characteristic traits like calcitonin, GRP and neurone-specific enolase production [11]. Array-CGH showed a balanced profile in this cell line and the existence of balanced structural chromosomal rearrangements, like translocations, could also be excluded by M-FISH. MTC can thus arise in the absence of chromosomal aberrations and without *RET* mutation [2].

TT cells were derived from a 77 year old female diagnosed with MEN2A [15], [16]. Besides structural abnormalities of chromosomes 1, 3, 4, 5, 7, 8, 10, 11, 12, 13, 14 and 20 [17], TT cells also hold a point mutation in the *RET* protooncogene (C634W) [18].

Normal human skin fibroblasts, HF-SAR, were established from skin of a two year old male (personal communication R. Pfragner) and served as control. The cells grow adherent and exhibit a fibroblastic morphology.

1.5 Shikonin and its derivatives

1.5.1 Historical Introduction

Extracts of the dried roots of Boraginaceae genera *Arnebia* and *Lithospermum*, otherwise known as *Zicao*, have been used in traditional Chinese medicine for thousands of years. Known as "shikon" in Japan, these herbal extracts have been prescribed for the treatment of macular eruptions, measles, sore-throat, burns and carbuncles. However, before *Zicao* was detected by physicians, extracts of the roots of *Lithospermum erythrorhizon* Sieb. et Zucc, *Arnebia euchroma* (Royle) Johnst or *Arnebia guttata* Bunge first captured the attention of botanists because they contain deep red pigments. Their use for dyeing silk probably dates back to centuries BC and it was not until two centuries AD that the great Chinese surgeon Hua To started appreciating the crude extracts for their antiseptic and anti-inflammatory properties [19], [20].

The active compounds of the extracted roots were identified as shikonin and alkannin derivatives by H. Brockmann [21] in 1936. He found that shikonin and alkannin were enantiomers and he correctly assigned their chemical structures. Later it was discovered that the amounts of alkannin, shikonin and their derivatives isolated from dried roots of Boraginaceae varied with the species of plant they were extracted from.

Even though *Zicao* has been applied in the treatment of all kinds of diseases continuously for millennia, the medicinal relevance of the active constituents of the extracts was confirmed experimentally only in 1978 by Papageorgiou [22]. Since then, biological investigations of the complete plant extract as well as its single components have shown that many of the properties claimed for Boraginaceae extracts in historical literature indeed have a scientific basis. Xin Chen summed up pharmacological studies conducted with shikonin derivatives until 2002 in a review article [23]. He gives an overview of the widely diverse activities attributed to shikonin and its derivatives, like wound healing, enhanced granuloma formation, suppression of local acute inflammatory reactions, inhibition of angiogenesis, inhibition of select chemokine ligands, inhibition of DNA topoisomerase activity, inhibition of platelet activation and antimicrobial activity, thereby coming to the conclusion that "shikonin is an effective inhibitor of protein-protein interaction with multiple targets in both the

intracellular and extracellular compartments". This hypothesis, however, has only partly been proven right as elaborated in the next section.

1.5.2 Biological investigations on shikonin between 2002 and 2011

Intriguingly, the search for elucidation of the molecular effects of shikonin continues up to date. Between 2002 and 2011 a number of contradictory phenotypic effects have been attributed to shikonin as summarized in Table 2.

Table 2 – Summary of phenotypic effects induced by shikonin

Effect		Citation
downregulation of	chemokine receptors, especially CCR5	[24]
	VEGFR2	[25]
	CDK4	[26]
	steroid sulfatase	[27]
	NF-κB	[28], [29], [30]
	MMP9	[30]
morphology of necrotic cell death		[31], [32]
loss of plasma membrane integrity		[31]
loss of mitochondrial membrane potential		[31], [33]
activation of autophagy		[31]
elevation of ROS		[29], [31], [33], [34]
induction of apoptosis		[26], [28], [29], [30], [33], [34], [35], [36]
inhibition of phosphorylation of (V)EGFR2		[25], [35]
	ERK	[25], [35],
inhibition of cell proliferation		[25], [26], [27], [28], [29], [31], [32], [33], [34], [35], [36]
cell cycle arrest		[26], [33], [34], [36]

During that time, attempts to identify the molecular target of shikonin have also been made. Chen, J. et al [32], for example, found that shikonin and its analogs directly target the tumor-specific pyruvate kinase-M2 and thereby inhibit the rate-determining step of glycolysis which is vital for cancer cell proliferation and survival.

Mao, X. et al [34], on the other hand, suggest a model where the application of shikonin leads to a rapid generation of ROS, which triggers JNK phosphorylation leading to mitochondrial dysfunction, cytochrome c release and caspase activation.

Another suggested mechanism was published by Wu, Z. et al [26]. They propose that shikonin induces DNA damage which in turn leads to cell cycle arrest due to p53 activation. Simultaneously, apoptosis is supposedly induced by downregulation of cyclin dependent kinase 4.

Finally, a completely different approach was presented by Yang, H. et al [37] when they found that shikonin inhibits tumor proteasome activity. According to their research the chymotrypsin-like activity of purified 20S and tumorcellular 26S proteasome is potently inhibited and computational modeling predicted that shikonin interacts with the catalytic site of the β5 chymotryptic subunit of the proteasome. They also report that treatment of cancer cell lines with shikonin lead to the accumulation of ubiquitinated and proteasome target proteins which induced cell death.

Considering all these different phenotypes and mechanisms published for shikonin derivatives, it can be assumed that only a part of the broad spectrum of molecular interactions has been discovered so far. An explanation for a more global mode of action is yet to be given.

1.5.3 The newest findings – the controversy continues through 2013

Two papers were published in late 2012 [38] and early 2013 [39], respectively, both claiming that they elucidated the "real" molecular target of shikonin and that all previously purported mechanisms of action should be viewed with caution. Interestingly enough, these two publications again have completely different outcomes.

Chen, C. et al [39] on the one hand report that shikonin is a cytotoxic DNA-binding agent in Ewing sarcoma cell lines. In a high-throughput screening for compounds disrupting the binding of transcription factors to DNA they found shikonin to be very effective. Using a SYBR green replacement assay they demonstrated that shikonin does not intercalate in DNA but much rather is a minor groove binder. Consequently they found that shikonin does not suppress gene expression in a global manner. They further hypothesized that shikonin, as common for DNA-binding compounds, has base pair preferences and thus only inhibits the transcription of certain genes. This way the downregulation of MMP9 expression previously described by Min, R. et al [30] is possibly explained.

Wiench, B. et al [38], on the other hand, claim that shikonin directly targets mitochondria and causes mitochondrial dysfunction. They performed microarray gene expression profiling of shikonin treated U937 lymphoma cells and found major cellular functions to be affected. Genetic network analysis allowed a division into four categories of genes: mitochondrial function, ROS induction and DNA damage, apoptosis and cell cycle arrest, and cytoskeleton and migration. They then proposed that mitochondria themselves are the direct target of the substance and performed cellular uptake and co-localization assays showing that shikonin accumulated in mitochondria within 10min of application. Subsequently, they demonstrated that the potential difference across the mitochondrial membrane was significantly reduced by shikonin itself and that cellular ROS levels were increased. Oxidative DNA damage was detected and intracellular calcium levels increased in a slow but continuous manner. Long-term treatment with shikonin induced cell-cycle arrest and apoptosis via the mitochondrial pathway. Cell migration was found to be inhibited because the tubulin network was disassembled. From their conclusion that shikonin immediately accumulates in mitochondria and the comparison with previously published literature [40] on redox cycling of naphthoquinones, they further hypothesized that shikonin, a substituted naphthoquinone, is metabolized to an unstable semiquinone by reductive enzymes, like mitochondrial NADH-ubiquinone oxidoreductase (complex 1), through a one-electron reduction reaction. In the presence of molecular oxygen, semiquinones enter a redox cycle leading to the reformation of the original quinone compound

with the generation of ROS as byproducts. The accumulation of these ROS then results in the depolarization of mitochondrial membranes and their breakdown, thereby releasing proapoptotic compounds. The detected oxidative DNA damage is also thought to be a consequence of ROS accumulation and supposedly induces cell-cycle arrest. Increased intracellular calcium concentrations apparently promote the disassembly of microtubules and thus inhibit migration of the cells.

In conclusion, Wiench et al inferred from their experiments that ROS induction is a primary and direct effect of shikonin and not a downstream effect mediated indirectly. And so continues the collective effort of scientists to elucidate the molecular mechanisms concerted by shikonin derivatives that eventually lead to tumor cell death.

1.5.4 Obtaining shikonin derivatives from dried root extracts of *O.paniculata*

Zicao is traditionally prepared from dried roots of the Boraginaceae *Arnebia* or *Lithospermum* [20]. Shikonin (**1**) is the major component of, for example, *Lithospermum erythrorhizon* [38] and thus it comes as no surprise that most of the research hitherto has been conducted with the unsubstituted shikonin derivative. In the present study, however, emphasis was put on the investigation of the main components of *Onosma paniculata* Bureau & Franchet (Fig. 3), acetylshikonin and β,β-dimethylacrylshikonin.

Fig. 3 – *ONOSMA PANICULATA* BUR. & FRANCH. Picture © Color pictorial handbook of toxic Chinese herbs. Zhang, Q.R. (1994) Tianjin Science and Technology Translation and Publishing Corporation, Tianjin, p.320.

The shikonin compounds used in this study were kindly provided by Nadine Kretschmer, Institute of Pharmacognosy, Karl-Franzens University, Graz, Austria [41], [42]. Dried roots of *O. paniculata* were acquired at a medicinal market in Kunming, China in 2003. A petroleum ether extract was prepared by Soxhlet extraction and evaporated to dryness under reduced pressure. Consequently, an aliquot was dissolved in methanol and fractionated by preparative HPLC. The different fractions obtained were characterized by ^{1}H and ^{13}C NMR and circular dichroism (CD) spectroscopic measurements and found to contain β-hydroxyisovalerylshikonin (**2**), acetylshikonin (**3**), β,β-dimethylacrylshikonin (**4**), and a mixture of α-methylbutyrylshikonin (**5**) and isovalerylshikonin (**6**). The chemical structures of the shikonin derivatives are shown in Fig 4. The experiments in the presented study were performed with the unfractionated petroleum ether extract, the compounds acetylshikonin and β,β-dimethylacrylshikonin and shikonin, which was purchased from Sigma (St. Louis, MO, USA) since it was not contained in the *Onosma paniculata* plant extract.

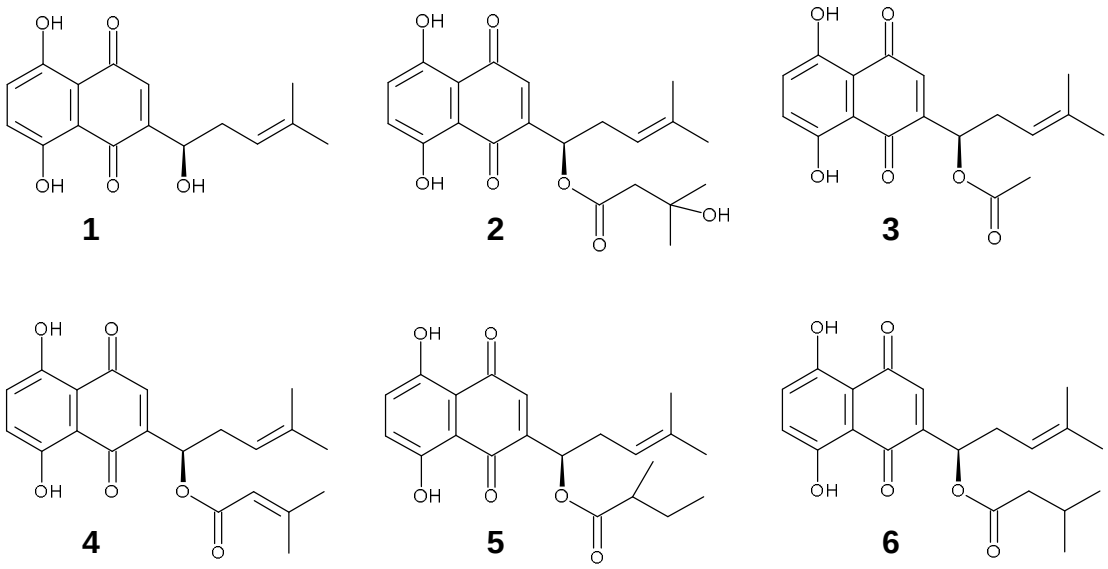


Fig. 4 – CHEMICAL STRUCTURES OF SHIKONIN DERIVATIVES. (**1**) shikonin, (**2**) β-hydroxy-isovalerylshikonin, (**3**) acetylshikonin, (**4**) β,β-dimethylacrylshikonin, (**5**) α-methylbutyrylshikonin and (**6**) isovalerylshikonin

1.6 Apoptosis

1.6.1 The biology of apoptosis

Apoptosis (greek: απόπτόσισ; after the "falling off" of leaves from trees) was first described by Kerr, J. et al [43] in 1972 as a basic biological phenomenon of controlled cell death. After investigating various tissues, he concluded that apoptosis plays a major role during normal embryonic development as well as in the turnover of healthy adult tissues. Electron microscopy revealed two discrete stages of the process: firstly, the breaking up of the cell into well-defined fragments and secondly, the degradation thereof after ingestion by surrounding cells.

Structural changes in cells undergoing apoptosis are described as condensation of both nucleus and cytoplasm – presumably as a consequence of the extrusion of water, formation of nuclear fragments and membrane blebbing. The separation of these protuberances formed on the cell surface leads to the formation of apoptotic bodies, which are compact, membrane-bounded cell remnants of great variation. Depending on the cellular constituents that happened to be present in the proximity of the bleb, apoptotic bodies differ in size and content. Small bodies may consist of condensed chromatin only, whereas others only comprise cytoplasmic elements [43].

The second step of programmed cell depletion involves the ingestion of the formed particles by cells of the surrounding tissue. After phagocytosis, apoptotic bodies are quickly transported to the lysosome, where they are degraded due to the prevailing pH and the activity of lysosomal hydrolases. These enzymes are acquired by fusion of pre-existing secondary lysosomes with the phagosome in the ingesting cell [43].

Apoptosis is continuously happening in normal fetal as well as healthy adult tissues, and was also found to be present in growing neoplasms. It is thought to be a mode of controlled cell death, playing a complementary but opposite role to mitosis, and thus contributing to the regulation of cell populations. There is no inflammation involved in this process and moreover, this mechanism is economical in terms of re-utilization of cellular components [43].

1.6.2 The biochemistry of apoptosis

1.6.2.1 Caspases: the "central executioners"

The main protagonists in the conduction of the observed morphological changes were found to be highly conserved cysteine-dependent aspartate-directed proteases, short caspases. All known caspases possess a cysteine residue in their active sites and cleave Asp-X-X-X motifs of their substrates. Different caspases show distinct specificities but they all cleave their substrates C-terminal of the aspartate residue [44].

As apoptosis is a highly regulated mechanism it comes as no surprise that caspases selectively cleave defined target proteins rather than just degrade all the protein present in a cell. In most cases, this proteolytic cleavage causes the inactivation of the substrate. Some targets, however, gain biological function through the removal of inhibitory domains or upon release from regulatory subunits. One example hereof is the activation of the nuclease that cuts genomic DNA between nucleosomes. Caspase-3 was found to cleave the inhibitory unit off this nuclease entitled CAD (caspase-activated DNase), resulting in the activation of the catalytic subunit. The active nuclease then degrades DNA into integer multiples of approximately 180 base pairs yielding in the so-called nucleosomal ladder which has been extensively used as marker for apoptotic cells [44].

Several other characteristic traits can also be explained by the caspase-mediated cleavage of target proteins. Nuclear shrinking and budding, for instance, are achieved by cleavage of nuclear lamins; cleavage of cytoskeletal proteins is responsible for the loss of overall cell shape and blebbing is induced by PAK2, which in turn is activated by caspase-mediated cleavage [44].

Over a dozen caspases have been identified in humans so far, all of them being homologous to each other. They are synthesized as zymogens (pro-caspases) consisting of three domains: the N-terminal prodomain, the p20 domain, and the p10 domain. Mature enzymes – generated through different mechanisms that are discussed below – all form heterotetramers consisting of two p20 and two p10 domains, and contain two active sites. In general, caspases can be divided in two groups according to their role during apoptosis: initiator and effector caspases. In addition to their different positions in the

apoptotic pathway, they also vary in the size of their N-terminal prodomains. Initiator caspases (CASP-2, -8, -9, -10) possess a longer prodomain than effector caspases (CASP-3, -6, -7) [44].

Although individual initiator caspases are activated through different mechanisms (see 1.6.2.2 and 1.6.2.3), they share the mode of action in which they activate effector caspases: by proteolytic cleavage of Asp-X sites – designated caspase cleavage sites – between the different domains. Exposure of procaspases to previously activated ones thus creates a cascade that allows for the rapid integration and amplification of pro-apoptotic signals [44].

Currently, two major pathways are described in the activation of apoptotic signaling. One involves binding of extracellular ligands to cell surface receptors and is therefore called "death-receptor pathway"; the other one is activated through intracellular signals and is executed through mitochondria, thus named "mitochondrial pathway". Both pathways converge at the level of effector caspase-3 (see Fig. 5) [44].

1.6.2.2 Induction of apoptosis via the death-receptor pathway

So far, five cell surface death receptors have been identified in mammalian cells, namely CD95, TNFR1, DR-3, DR-4 and DR-5 (see Table 3). They all belong to the tumor necrosis factor / nerve growth factor receptor superfamily. They are transmembrane proteins that share a conserved cysteine-rich repeat in the extracellular domain [45].

Table 3 – Death receptors and their ligands

Receptor	Receptor Synonyms	Ligand	Ligand Synonyms
CD95	Fas, APO-1	CD95L	FasL
TNFR1		TNF	
DR-3	Apo-3, WSL-1, TRAMP	Apo3L	
DR-4	TRAIL-R1	TRAIL	Apo2L
DR-5	TRAIL-R2, TRICK2, KILLER	TRAIL	Apo2L

Activating ligands of the cell surface death receptors are structurally related molecules belonging to the tumor necrosis factor gene superfamily, like CD95L, TNF, Apo3L and TRAIL [45].

Induction of apoptosis through the death-receptor pathway is thought to occur in a three-step process: binding of a ligand to the corresponding receptor leads to the trimerization thereof; this in turn allows for the recruitment of adaptor proteins on the cytosolic part of the membrane, which then activate initiator caspases [45].

The recruitment of adaptor molecules – also known as intracellular receptor-associated proteins – is facilitated by conserved domains in the cytosolic portion of the transmembrane receptors. These domains, termed death domains (DD), consist of 68 amino acids and are highly conserved. The recruited adaptor molecules, e.g. FADD (Fas-associating protein with death domain) or TRADD (TNF-receptor associated death domain), also contain this amino acid sequence at their C termini. The interaction between receptor and adaptor protein through physical interaction of death domains allows for the formation of the so-called DISC (death-inducing signaling complex). The N termini of adaptor proteins also contain a conserved domain, termed death effector domain (DED), since its function is the recruitment of procaspase-8 molecules to the afore formed DISC. Procaspase-8 contains two DEDs that interact with the DEDs of the recruiting adaptor proteins. This way, a relatively high local concentration of zymogen is achieved and it is believed that the low but present intrinsic protease activity of procaspase-8 leads to the cleavage of few initiator caspases, thereby activating them and allowing them to further activate each other and exert downstream effects [45].

The regulation of death receptor activity is achieved through three distinct mechanisms. The first mechanism involves proteins that prevent recruitment of procaspase-8 to the DISC by competing for the DED of adaptor proteins. An example for such an endogenous inhibitor is c-FLIP, cellular FADD-like ICE inhibitory protein. The second mechanism consists in the sequestration of ligands from their designated receptors by decoy receptors. The extracellular domains of decoy receptors are structurally very similar to those of their counterparts allowing them to bind the respective ligand. However, the signal is not transmitted onto the apoptotic pathway since decoy receptors lack the

cytosolic domain. This mechanism has been identified for TRAIL and its receptor and seems to play an important role in tumorigenesis. The third mechanism found in the regulation of death receptor induced apoptosis so far consists of the direct inhibition of the autoproteolytic activity of procaspase-8 by proteins like crmA [45].

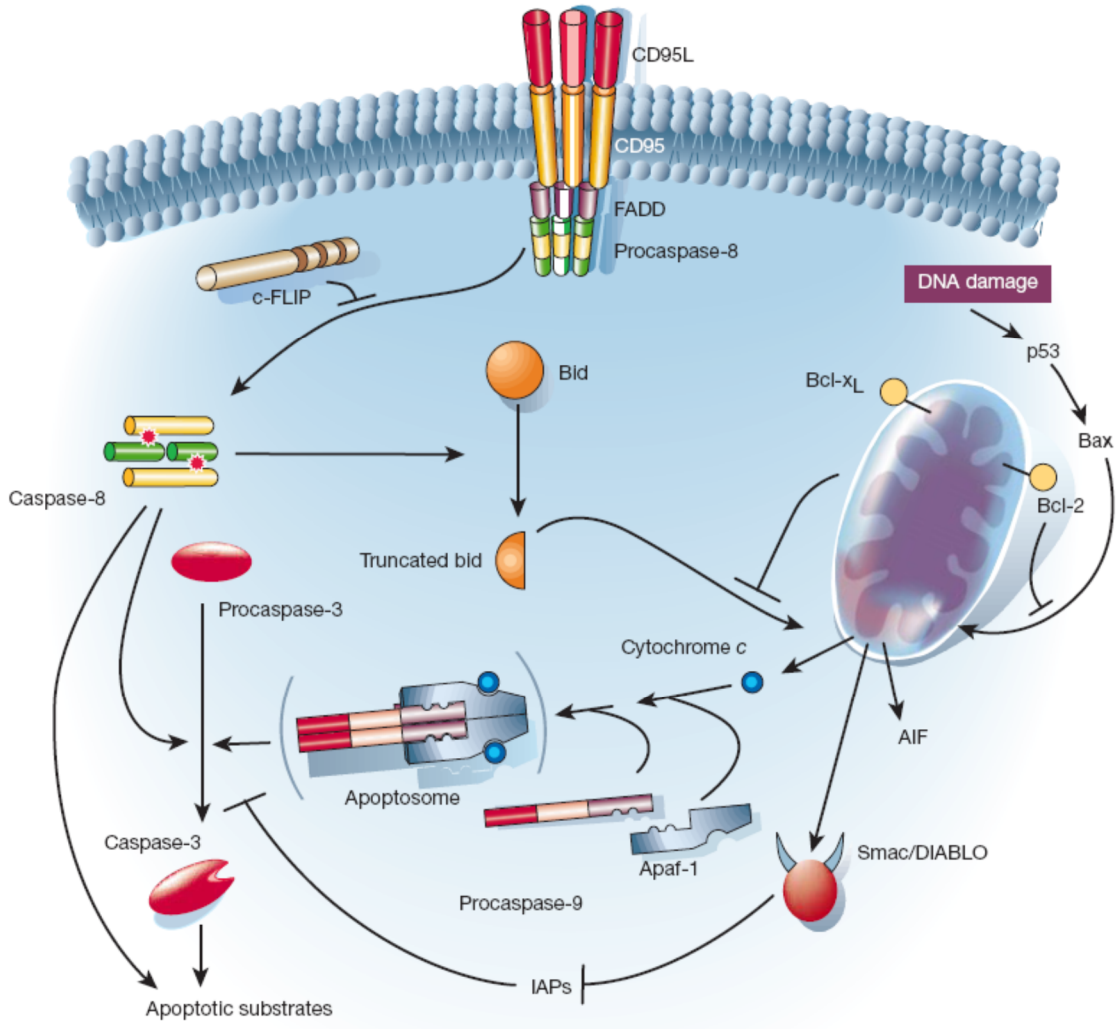


Fig. 5 – INDUCTION OF APOPTOSIS IN MAMMALIAN CELLS.
©Hengartner, M.O. *The biochemistry of apoptosis.* Nature (**2000**), *407*, p.773.

1.6.2.3 Induction of the mitochondrial pathway of apoptosis

Three proteins have been identified as key factors in the execution of the mitochondrial pathway of apoptosis: cytochrome c, Apaf-1 (Apoptotic protease activating factor 1) and procaspase-9 [44].

Caspase-9 is the initiator caspase in this pathway and its main function is the cleavage of procaspase-3 for apoptotic signal enhancement. (Pro-)caspase-9 contains a caspase activation and recruitment domain (CARD) which allows for interaction with other proteins. In contrast with all the other caspases studied so far, caspase-9 is not activated by proteolytic cleavage but by means of conformational change. This conformational change is achieved by the formation of the apoptosome (see Fig. 5), a complex that is formed as follows: hydrolysis of dATP to dADP drives the oligomerization of Apaf-1 which then allows for the binding of various cytochrome c molecules, and subsequently the recruitment of multiple procaspase-9 molecules via the CARD. Through this process, caspase-9 becomes activated and is now able to proteolytically cleave caspase-3 [45].

The release of cytochrome c, normally part of the electron transport chain and residing in the intermembrane space of mitochondria, to the cytosol is still under investigation. Presently, three basic models have been proposed. One particular family of proteins, the Bcl-2 family, appears to play a key role in all of them. Bcl-2 proteins (named after the first isolated member of the family, a gene involved in B-cell lymphoma) have a hydrophobic C-terminal tail that allows their localization to the outer mitochondrial membrane, with the remainder of the protein facing the cytosol. They can be roughly divided into two groups; anti-apoptotic factors as Bcl-2, Bcl-xL and Mcl-1, and pro-apoptotic factors like Bad, Bax, Bak, Bik, Bim and Bid. Although it is well known that members of both groups interact with each other through the formation of dimers and thereby neutralize each other, it is also commonly acknowledged that there must be more to it [46].

The first hypothesis [44] is based on the observation that Bcl-2 family members are able to integrate into lipid bilayers and form channels. It was thus concluded that Bcl-2 proteins might form channels in the outer mitochondrial membrane, similar to the ones formed by the diphtheria toxin, which allow cytochrome c to be released from the intermembrane space.

The second hypothesis suggests that Bax modulates the size of existing ion channels like, for example VDAC or ANT, and hence facilitates the transport of cytochrome c across the outer membrane.

The third hypothesis states that Bcl-2 proteins are able to influence the homeostasis of mitochondria. In this model, changes of the mitochondrial physiology cause a swelling of the inner membrane which results in rupture of the outer membrane, thereby releasing intermembrane proteins through lesions in the lipid bilayer.

Even though many questions concerning the mode of action still need to be answered, it cannot be overlooked that cytochrome c release from mitochondria plays an important role in the activation of the apoptotic cascade.

Interestingly, not much information concerning the regulation of Bcl-2 family members is available yet. Whereas some members seem to spend their entire lifespan attached to membranes, others are able to move between the cytosol and organelles. The cytosolic reservoir of inactive Bcl-2 proteins can be re-directed to mitochondria upon internal apoptotic signals like DNA damage [46]. Activation of the pro-apoptotic compounds then leads to the battle for cell's fate.

1.6.2.4 Regulation of apoptosis

The release of cytochrome c and other pro-apoptotic factors from mitochondria and their ability to amplify apoptotic signals by activating Bcl-2 proteins, and thus causing even more mitochondrial damage, seems like a way of making sure that the activation of the apoptotic cascade is a one-way road. However, such powerful positive feedback loops also require the presence of dampeners. One family of proteins that act as such dampeners are inhibitors-of-apoptosis (IAP). Without their presence, even the smallest cytochrome c leakage from mitochondria would result in the full activation of the apoptotic machinery. IAP proteins, on the other hand, are regulated by IAP inhibitors known as Smac (second mitochondria-derived activator of caspases) or DIABLO (direct IAP-binding protein with low pI). Interestingly, Smac/DIABLO only gets released

from mitochondria upon activation of the mitochondrial pathway, as if to make sure that cells induced to die are not stopped by IAP proteins [44], [46].

In conclusion, activation of the apoptotic pathway results in the activation of effector caspases, which subsequently cleave other substrates. This leads to characteristic morphological changes like chromatin condensation, nucleosomal DNA fragmentation, mitochondrial membrane breakdown, flipping of phosphatidylserine residues from the cytosolic to the extracellular side of the plasma membrane, and the formation of apoptotic bodies.

2. AIM OF THIS THESIS

The aim of the present study was to investigate the effect of

- an unfractionated petroleum ether extract of the roots of *Onosma paniculata* and
- the pure derivatives shikonin, acetylshikonin and β,β-dimethylacryl-shikonin

on

- medullary thyroid carcinoma cell lines MTC-SK, SHER-I and TT and
- normal human skin fibroblasts, HF-SAR, serving as control.

Up to date, shikonin has been tested on several cell lines derived from various human tumors [26], [28], [29], [33]–[38], [47], [48]. In the present study, we investigated for the first time the effect on cell lines derived from medullary thyroid carcinomas. Additionally, the efficacy of shikonin was compared to that of its derivatives acetylshikonin and β,β-dimethylacrylshikonin. Our working hypothesis was that shikonin derivatives induce apoptosis in cancer cell lines but not in normal human skin fibroblasts.

Concerning the underlying mechanism, we aimed to examine whether migration and invasion of cancer cells were reduced through down-regulation of MMP9, as claimed by Min, R. et al [30].

3. MATERIALS AND METHODS

3.1 Tumor collection and freezing

In November 2002 tumor tissue was surgically removed by Univ.-Prof. Dr. Bruno Niederle (Section Surgical Endocrinology, Medical University of Vienna) from a male patient diagnosed with familial medullary thyroid carcinoma (FMTC). Molecular genetic studies revealed a point mutation in exon 10 of the *RET* protooncogene (TGC>TAC). The specimen was placed in 10 ml transport medium (Ham's F12 supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin) and delivered to the cell culture laboratory within 24 hours. There, the specimen was soaked in concentrated antibiotic solution (1000 U/ml penicillin and 1000 µg/ml streptomycin in HBSS) for 20 minutes at room temperature. Fat and visible connective tissues were removed and the tumor tissue was transferred to HBSS and cut into pieces of approximately 3 to 4 mm diameter. Some pieces of the biopsy were preserved by freezing. Therefor growth medium (Ham's F12 supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin) was complemented with 10% DMSO. Four to five pieces of the tumor tissue were added to 1 ml of freezing medium in a cryotube, which was then slowly frozen at 1°C/min and finally stored in liquid nitrogen.

3.2 Primary culture of a neuroendocrine tumor

Ten years after the surgical removal, one cryotube containing two pieces of tumor tissue with an approximate size of 0.5mm³ each was thawed. A primary culture encoded "WEAL" was set up according to protocol [14]. After quickly thawing the specimen, the biopsy was further cut into pieces using surgical scissors. The suspension was then transferred to a centrifuge tube using a pipette with a wide tip (i.e., an old glass pipette with a broken tip) and centrifuged at 300xg for 5 min. After discarding the supernatant, the sample was resuspended in growth medium (Ham's F12: M199, 1:1, supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin; all from PAA Laboratories, Pasching, Austria) and cultures were set up in tissue culture flasks. For better adhering of the cells, flasks had previously been coated with

poly-D-lysine (BD, Vienna, Austria). Samples were incubated in a separate incubator, which is designated for primary cultures only. After three days, flasks were checked for contamination and a small amount of medium was added.

3.3 Maintaining primary cultures

Over the following weeks cultures were observed and supplied with fresh medium according to necessity. In the beginning some cells attached to the culture vessel and eventually a monolayer containing mixed populations of epithelioid cells, fibroblasts, and others was formed. At the initial stage of cultivation, the cell number is usually low and a complete medium change would subduct the cultures of valuable autocrine or paracrine growth factors. Hence, only partial replacement of medium is recommended: half of the medium containing floating cells was withdrawn, centrifuged (300xg, 5min), and the pelleted cells were resuspended in fresh medium and either returned to the original vessel or collected in a new vessel preferably coated with poly-D-lysine. These steps were repeated over weeks, until multicellular spheroids arose from the monolayers.

When fibroblastic overgrowth occurred monolayers were treated with collagenase-medium (growth medium containing 0.12 units/ml collagenase type 2). Collagenases are enzymes, namely matrix metalloproteases that cleave peptide bonds of collagen, which is produced by fibroblasts and constitutes the stroma of animal tissue and thus cancer tissue as well. This way cancer cells can be separated from the remaining cells for further cultivation [14].

In order to avoid cross contamination, as described by W. Nelson-Rees in 1981 [49], an individual medium bottle was prepared for each cell line kept in culture.

3.4 Cell lines and cell culture

TT cells, initially acquired from ECACC (Porton Down, Salisbury, UK), were cultured in T75 tissue culture flasks (Sarstedt, Wr. Neudorf, Austria) with Ham's F12 Nutrient Mixture (Sigma Aldrich, Steinheim, Germany) supplemented with 10% FBS (Fetal Bovine Serum Gold; PAA Laboratories, Pasching, Austria). Cells were passaged at approximately 80% confluence to an initial cell number

of $2x10^5$ cells/ml to using trypsin-EDTA (PAA Laboratories, Pasching, Austria). Passage numbers of TT cells used for the experiments were 59 to 75.

The human medullary thyroid carcinoma cell lines MTC-SK [12] and SHER-I [11] and the human skin fibroblast cell line HF-SAR [50] were established in our laboratory. Suspension cells MTC-SK and SHER-I were cultured in Ham's F12:M199 medium 1:1 (PAA Laboratories, Pasching, Austria) supplemented with 10% FBS. Cells were passaged once a week to an initial cell number of $2x10^5$ cells/ml. Experiments were conducted with passage numbers 101 to 123 for MTC-SK cells and 58 to 78 for SHER-I cells.

Normal human skin fibroblasts, HF-SAR, were cultured in Eagle's minimum essential medium with non-essential amino acids and sodium pyruvate (EMEM; BioWhittaker, Lonza, Verviers, Belgium) supplemented with 2mM L-glutamine (PAA Laboratories, Pasching, Austria) and 10% FBS. Cells were passaged to an initial cell number of $1x10^5$ cells/ml using accutase (PAA Laboratories, Pasching, Austria) according to the manufacturer's protocol. For the experiments, cells at passage numbers 12 to 27 were used.

All cells were maintained in a humidified 5% CO_2 atmosphere at 37°C.

3.5 Shikonin derivatives

Acetylshikonin (AS), β,β-dimethylacrylshikonin (DMAS), shikonin (Shik; Sigma-Aldrich, St. Louis, MO, USA) and a petroleum ether extract (EX) of the roots of *Onosma paniculata* were kindly provided by the Department of Pharmacognosy, Institute of Pharmaceutical Research, Karl-Franzens University, Graz, Austria. Aliquots of the substances were freshly dissolved in DMSO (Dimethyl Sulfoxide Hybri-Max®; Sigma, Vienna, Austria) every two weeks to ensure consistent bioactivity throughout the entire project. For every compound a stock solution of 10 mg/ml was prepared and diluted subsequently with DMSO. Whenever a substance was added in an experiment, it was pipetted from the stock with the highest possible concentration in order to keep the amounts of DMSO added to the cell culture medium as low as possible. In any case, the final concentration of DMSO never exceeded 0.5% which has been shown to have no effect on cell proliferation, viability or morphology.

3.6 Growth inhibition assay

Cells were centrifuged at 300xg for 5min after multicellular aggregates of suspension cells MTC-SK and SHER-I were pipetted into single cells. The supernatant medium was removed and the cell number was adjusted to $2x10^5$ cells/ml in fresh medium. Substances were added in at least five different concentrations and aliquots of 0.9ml were seeded into 24-well plates (Sarstedt, Wr. Neudorf, Austria). After incubation in humidified 5% CO_2 atmosphere at 37°C for 24, 48 and 72 hours, respectively, cell clusters were pipetted into single cells again and counted in triplicates using a CASY1® Cell Counter & Analyser TTC (Schärfe System, Reutlingen, Germany).

Adherently growing TT cells were harvested after trypsinization, centrifuged, and resuspended in fresh medium. Cell number was adjusted to $2x10^5$ cells/ml and aliquots (2ml) were seeded into 6-well plates (Sarstedt, Wr. Neudorf, Austria). After allowing the cells to adhere for 18 hours, at least 7 different concentrations of each substance were added. 72 hours later, cells were washed with 1ml of PBSA[1] and treated with 500µl trypsin-EDTA for ~2min. Cells were counted in triplicates after the addition of 1.5ml of FBS containing medium.

All assays were performed with at least three different passage numbers and with medium containing 10% FBS, but no antibiotics.

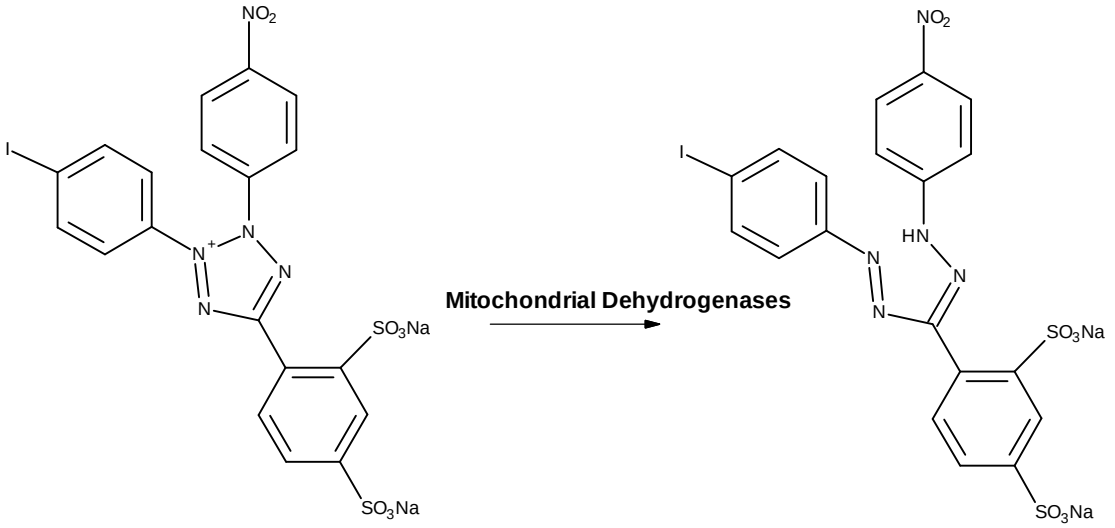
3.7 Determination of IC_{50} values

Cell numbers measured in the above mentioned growth inhibition assay were plotted against the applied concentrations of shikonin derivatives using Microsoft Excel (Office 2010). IC_{50} values represent the concentrations required to reduce cell proliferation to 50% and were determined by linear regression. Results are presented as mean ± SD of at least three independent experiments.

[1] PBSA: Dulbecco's phosphate buffered saline lacking Ca^{2+} and Mg^{2+} [4]; 136.9 mM NaCl, 2.7 mM KCl, 8.0 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 11mM dextrose

3.8 Cell viability assay

Cell viability was assessed by a colorimetric assay based on the cleavage of the water soluble tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate). WST-1 is cleaved to formazan by mitochondrial dehydrogenases (Fig. 6). An increase in the number of viable cells leads to an increase in the overall activity of cellular enzymes. This gain in enzyme leads to an increase in the amount of formazan dye formed, which directly correlates with the number of metabolically active cells. Measuring the absorbance of the formazan dye with a spectrophotometer allows quantification of the metabolically active cells [51].



WST-1 **Formazan**

Fig. 6 – CLEAVAGE OF THE TETRAZOLIUM SALT WST-1 (modified from [51])

Aliquots (1.2 ml) of MTC-SK and SHER-I cell suspensions ($2x10^5$ cells/ml) were seeded into 24-well plates after supplementation with DMSO (solvent control) or appropriate concentrations of shikonin derivatives. Cell viability was measured after 24, 48 and 72 hours of incubation, respectively, by pipetting cell aggregates into single cells, transferring 100µl of cell suspension into 96-well plates (Sarstedt, Wr. Neudorf, Austria) and adding 10µl of WST-1 reagent (Roche Diagnostics, Mannheim, Germany). After an additional incubation period of approximately 2 hours at 37°C, 5% CO_2, the absorbance of the samples was measured at 450nm with a reference wavelength of 650nm ($Thermo_{max}$ microplate reader, Molecular Devices, Ramsey, MN, USA).

Adherently growing cells TT and HF-SAR were directly seeded into 96-well plates in aliquots of 100µl with cell densities of $1x10^5$ cells/ml and $3x10^4$ cells/ml, respectively. Cells were allowed to adhere for 18 hours before the medium was removed and exchanged for medium supplemented with DMSO (solvent control) or different concentrations of shikonin derivatives as indicated. Cell viability was again measured after 24, 48 and 72 hours, as described above.

All samples were tested in 6 replicates and 100µl of medium plus 10µl of WST-1 reagent served as blank. Results are presented as percentage of solvent (DMSO) treated control cells.

3.9 Cell morphology

Morphological changes occurring in the cell populations after application of shikonin derivatives were observed with a Nikon inverted microscope (Eclipse TE 300, Nikon, Tokyo, Japan). Suspension cells MTC-SK and SHER-I were pipetted into single cells ($2x10^5$ cells/ml), supplemented with shikonin derivatives AS, DMAS, Shik and EX according to their IC_{50} values and seeded into 24-well plates (0.5 ml per well). Aliquots (2ml) of adherent TT cells were seeded into 6-well plates at a density of $2x10^5$ cells/ml. After allowing them to adhere overnight, they were incubated with the corresponding IC_{50} values of the shikonin derivatives. Cell morphology was observed after 24, 48 and 72 hours. Images were taken with a Nikon 12-bit CCD camera (Nikon, Tokyo, Japan).

3.10 Monolayer wound healing assay

The monolayer wound healing assay mimics to some extent the migration of cells *in vivo*. This method is based on the observation that cells move over a created gap, the so called “scratch”, in order to reestablish cell-cell contacts [52]. TT cells (2 ml) were seeded into 6-well plates at a density of $5x10^5$ 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 1ml PBSA which was then replaced by 2ml medium supplemented with either DMSO (solvent control) or the corresponding $IC_{50}/5$ values of the shikonin derivatives. Reference points

were created by marking the lid of the plate and images were acquired using a phase-contrast microscope and a 12-bit CCD camera (Nikon, Tokyo, Japan). Cells were incubated at 37°C, 5% CO_2 and observed periodically until closure of the scratch was achieved by the control cells. Experiments were performed with three different passages and analysis was done using TScratch software [53]. Statistical analysis was done with a two-tailed unpaired Student's t-test where p-values <0.05 were considered significant and p-values <0.01 as very significant. Results are shown as mean value ± standard deviation.

3.11 Matrigel™ invasion assay

Through the Matrigel™ Invasion Assay cell invasion of malignant and normal cells can be studied *in vitro*. Adherently growing cells were placed on an insert containing an 8 micron pore size PET membrane covered with a thin layer of Matrigel™ Basement Membrane Matrix (BD Biosciences, Erembodegem, Belgium). The matrix resembles the basement membrane *in vivo* and occludes the pores of the membrane, thereby blocking non-invasive cells from migrating through the membrane. Invasive cells on the other hand are able to detach themselves from and invade through the matrix and the membrane. They can then be visualized and counted [54].

For rehydration of the BD BioCoat™ Matrigel™ Invasion Chamber 0.5ml of Ham's F12 medium without serum were added to both the well and the insert. After incubation at 37°C, 5%CO_2 for at least 2 hours the medium was removed carefully. 750µl of Ham's F12 containing 10% FBS were added to the wells and $1x10^5$ cells (in 500 µl Ham's F12 supplemented with DMSO as solvent control or certain concentrations of shikonin) were placed on top of the insert. After incubation for 48 hours at 37°C, 5% CO_2 the medium was aspirated and the Matrigel™ removed by scrubbing with a dry cotton swab. A cotton swab moistened with PBSA was used to clean the membrane from any remainders of the matrix. The membrane was then washed with PBSA and the invaded cells were fixed by placing the membrane in 96% EtOH for 5min. After washing with PBSA again, the membrane was incubated in a solution of 1µg/ml DAPI (4',6-diamidine-2'-phenylindole dihydrochloride; Roche Diagnostics, Vienna, Austria) in PBSA for 10min. DAPI is a fluorescent dye that binds to DNA and therefore

allows the visualization of cell nuclei. After thoroughly washing the membrane a scalpel was used to carefully cut it from the insert and transfer it onto a glass slide. Invaded cells were analyzed by fluorescence microscopy (Leica DM 4000B; Leica, Wetzlar, Germany), images were acquired using a DFC300 FX camera (Leica, Wetzlar, Germany) and cell numbers were determined with CellC software [55].

3.12 Hoechst staining

2'-(4-Ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2'5'-bi-1H-benzimidazoletrihydro-chloride (Hoechst 33342) is a specific fluorescent DNA stain that binds to the minor groove of AT-rich regions. Treatment of cells with the fluorophore leads to a blue coloring of the nuclei when excited with UV light. The stain can be used to visualize the morphology of nuclei of living cells. While untreated, healthy cells show regular round nuclei, cells that undergo apoptosis can be identified by the condensed chromatin and fragmented morphology of the nucleic bodies. Suspension cells MTC-SK and SHER-I were supplemented with shikonin derivatives AS, DMAS, Shik and EX according to their IC_{50} values and aliquots (1ml) with a cell number of $2x10^5$ cells/ml were seeded into 24-well plates. After incubation periods of 24 and 48 hours, respectively, cell clusters were pipetted into single cells and Hoechst 33342 (Cell Signaling Technology, Danvers, MA, USA) was added to a final concentration of 1µg/ml. Cells were harvested by centrifugation (5min, 300xg), resuspended in 10µl PBSA, pipetted onto glass slides, covered with a cover slip and analyzed under a fluorescence microscope (Leica DM 4000B; Leica, Wetzlar, Germany). The microscope was controlled with Leica microsystems software and pictures were acquired with a DFC300 FX camera (Leica, Wetzlar, Germany) using an A4 filter cube.

3.13 Caspase-Glo® 3/7 assay

Effector caspases 3 and 7 recognize tetrapeptide motifs Asp-x-x-Asp and hydrolyze peptide bonds after the C-terminal aspartic acid residue. The Caspase-Glo® 3/7 Assay provides a luminogenic substrate, which contains the tetrapeptide sequence DEVD, luciferase and cell lysis agents. Addition of the reagent to cell suspensions leads to cell lysis, followed by caspase cleavage of

the substrate and generation of a luminescent signal (Fig. 7), which is proportional to caspase activity and can be detected with a luminometer [56].

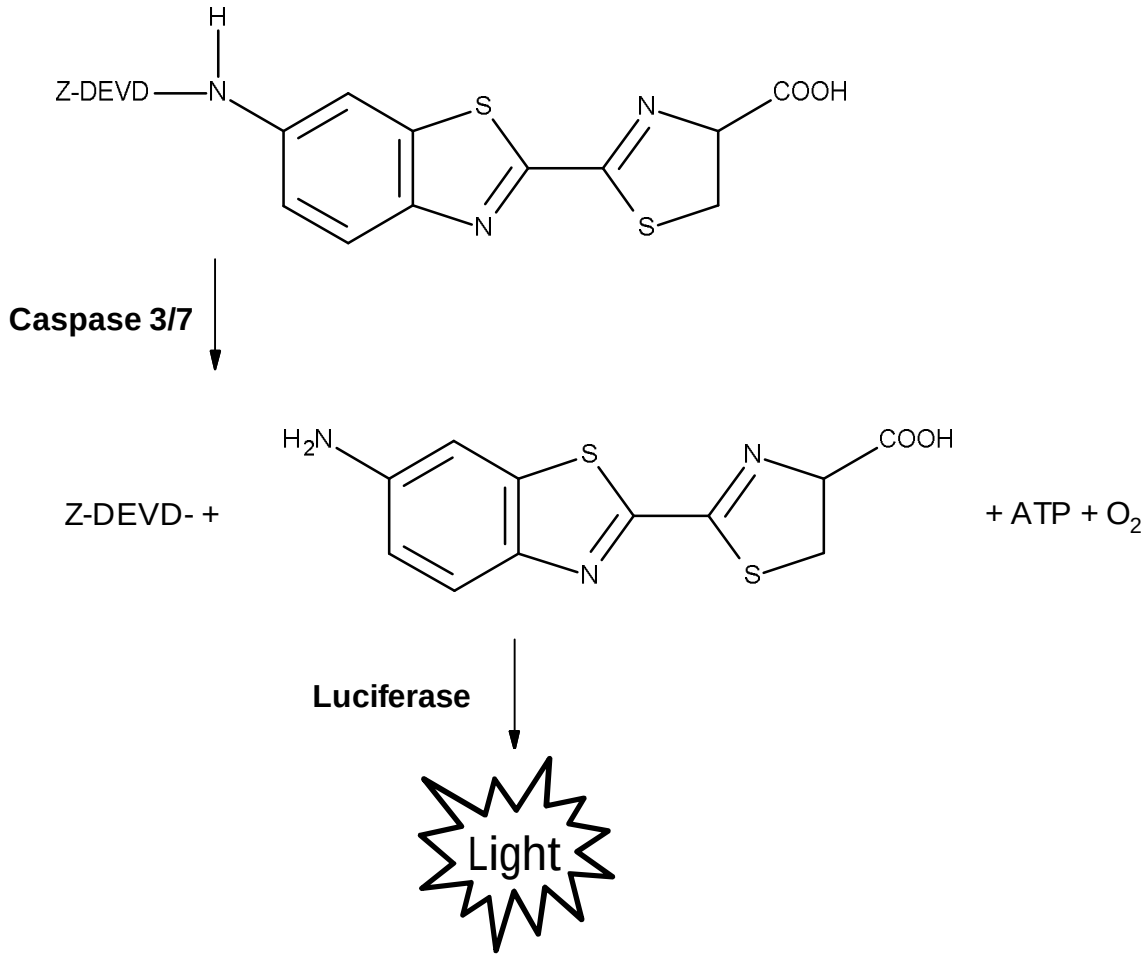


Fig. 7 – CASPASE-GLO® 3/7 ASSAY (modified from [56])

Suspension cells MTC-SK and SHER-I were supplemented with DMSO as solvent control or shikonin derivatives AS, DMAS, Shik and EX according to their IC_{50} values. Aliquots (0.9ml) were seeded into 24-well plates at a cell number of $2x10^5$ cells/ml and incubated for 6 and 12 hours, respectively. After the incubation period, cell suspensions were allowed to cool to room temperature. Cell clusters were then pipetted into single cells and 50µl were transferred into white-walled 96-well luminometer plates (Nunc™, Thermo Fisher Scientific, Vienna, Austria). 50µl of the Caspase-Glo® 3/7 reagent (Promega, Madison, WI, USA) were added and the samples were incubated for 30min at room temperature in the dark. Luminescence was then measured with a GloMax®-Multi+ Detection System (Promega, Madison, WI, USA). All samples were tested in 5 replicates and 50µl medium plus 50µl Caspase-Glo® 3/7

reagent served as blank. Results are presented normalized to solvent control (DMSO) treated cells.

3.14 Evaluation of apoptosis by flow cytometry

Phosphatidylserine (PS) residues are normally hidden within the plasma membrane and only flip to the surface of the cell when apoptosis is induced. *In vivo*, the presence of PS on the cell surface is a specific signal for macrophages to recognize and remove apoptotic cells. *In vitro*, the anticoagulant Annexin V can be used to detect cellular changes since it shows a high affinity for binding to PS. As apoptotic processes progress, the cell membrane becomes permeable for 7-Amino-Actinomycin D (7-AAD), which is a DNA specific dye. By staining treated and untreated samples of cells with Annexin V and 7-AAD it is possible to distinguish healthy, early and late apoptotic, and dead cells from each other [57].

$1x10^6$ MTC-SK or SHER-I cells were seeded at a density of $2x10^5$ cells/ml into T25 flasks and supplemented with DMSO (solvent control) or different concentrations of acetylshikonin and shikonin. After incubation at 37°C, 5% CO_2 for various times, cells were harvested by centrifugation (300xg, 5min), washed with PBSA and resuspended in Annexin binding buffer (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. Cell numbers of all samples were counted with a CASY®1 Cell Counter & Analyzer and $1x10^5$ cells were transferred to a FACS tube (BD Biosciences, Erembodegem, Belgium). After adjusting the volume to 100µl with Annexin binding buffer, 5µl Annexin V-FITC (BD Biosciences, San Jose, CA, USA) and 5µl 7-AAD (BD Biosciences, San Jose, CA, USA) were added. The samples were thoroughly homogenized by pipetting and kept on ice for 20min in the dark. Subsequently, 400µl of Annexin binding buffer were added and $1x10^4$ events were analyzed by flow cytometry (LSRFortessa; BD, Biosciences, San Jose, CA, USA), respectively.

3.15 Cell cycle analysis by flow cytometry

$2x10^6$ MTC-SK or SHER-I cells were incubated with either DMSO (solvent control) or the corresponding IC_{50} and $2xIC_{50}$ concentrations of shikonin and

acetylshikonin for 24 hours. Cells were harvested by centrifugation (300xg, 5min) and washed with BD Cycletest™ Buffer Solution (BD, Erembodegem, Belgium). $5x10^5$ cells of each sample were then transferred into FACS tubes, centrifuged (300xg, 5min) and either fixated in 75% EtOH and stored at -20°C, or directly processed. Cells were stained with propidium iodide according to the manufacturer's protocol (BD, Erembodegem, Belgium). $2x10^4$ events were analyzed by flow cytometry (LSRFortessa; BD, Biosciences, San Jose, CA, USA), respectively.

3.16 Analysis of mitochondrial transmembrane potential

The effect of acetylshikonin and shikonin on the mitochondrial transmembrane potential (ΔΨm) was analyzed by JC-1 (Cayman, Tallinn, Estonia) staining. During apoptosis, several events occur in mitochondria, including the release of caspase activators, changes in electron transport, and loss of the transmembrane potential. JC-1 is a lipophilic cation that selectively enters mitochondria. Aggregates that exhibit a red fluorescence are formed inside healthy mitochondria whereas depolarization of the transmembrane potential leaves JC-1 in a monomeric state emitting green light. Induction of apoptosis thus causes a shift from red to green fluorescence.

Aliquots (1ml) of MTC-SK and SHER-I were seeded at a density of $2x10^5$ cells/ml and incubated with JC-1 according to the manufacturer's protocol. Acetylshikonin and shikonin were then added at the corresponding IC_{50}, $2xIC_{50}$ and $4xIC_{50}$ concentrations. After 6 hours of incubation, cells were analyzed by flow cytometry (LSRFortessa; BD, Biosciences, San Jose, CA, USA).

3.17 Isolation of RNA

$1x10^6$ cells (MTC-SK or SHER-I) were seeded at a density of $2x10^5$ cells/ml, supplemented with either DMSO (solvent control) or the concentrations of acetylshikonin and shikonin equivalent to their IC_{50} values. Cells were harvested after 4 or 24 hours of incubation at 37°C, 5%CO_2 by centrifugation (300xg, 5min). Lysis of the cells was accomplished by addition of 1ml TRI Reagent® (Molecular Research Centre, Cincinnati, OH, USA) and subsequent homogenization by pipetting. After 5min of incubation at room temperature,

100µl of 1-bromo-3-chloropropane were added; the test tubes were inverted for 20 times and incubated for another 10min at room temperature. Phase separation was obtained by centrifugation (15min, 12000 g, 4°C) and the upper phase was transferred to a new Eppendorf tube. 500µl of isopropanol were added, the tubes were inverted 20 times again, and incubated for 10min at room temperature. The RNA precipitated and the supernatant was removed after centrifugation (8min, 12000 g, 4°C). After washing the RNA with 1ml ice cold 75% EtOH (centrifugation for 5min, 7500 g, 4°C) the pellet was left to dry for approximately 3min. Subsequently, 50µl of RNase free H_2O were added and the RNA was brought into solution by heating (55°C, 10min).

RNA concentrations were determined in triplicate using a NanoDrop® spectrophotometer (ND-1000; peQLab Biotechnology, Erlangen, Germany) and samples were diluted to 100ng/µl. Only samples with an OD_{260}/OD_{280} ratio between 1.8 and 2.0 were further processed.

RNA quality was examined by gel electrophoresis. Therefore, 500ng of isolated RNA were submitted to agarose gel electrophoresis (1% agarose, 80V, 45min). TAE buffer was used for pouring and running the gel. Samples were considered of quality if distinct 28S and 18S rRNA bands were visible and no smeer occurred.

3.18 Reversed transcription of RNA to cDNA

1000ng of total RNA were converted to cDNA by reverse transcriptase using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Synthesis of cDNA is initiated by random hexamer primers that ensure that the first strand synthesis occurs efficiently with all species of RNA molecules present. Thermal cycling conditions (S1000™ Thermal Cycler; BioRad, Vienna, Austria) were as recommended by the manufacturer: 25°C (10min), 37°C (120min), 85°C (5sec), 4°C (∞).

3.19 Quantification of cDNA by real-time PCR

Quantification of gene expression was done by real-time PCR using iQ™ SYBR® Green Supermix (Bio-Rad, Vienna, Austria) which contains iTaq DNA polymerase, dNTPs, $MgCl_2$, SYBR® Green dye, enhancers, stabilizers and

fluorescein. The reaction mixture was prepared with primers (Table 4) having a final concentration of 0.2µM and 20ng cDNA were added to each well. The final volume of each sample was adjusted to 15µl with nuclease free H_2O. The following thermal cycling conditions (C1000^{TM} Thermal Cycler; Bio-Rad, Vienna, Austria) were applied: initial denaturation at 95°C for 3min followed by 40 cycles consisting of strand separation at 95°C for 10sec, annealing at 61.5°C for 40sec and extension at 72°C for 15sec. After PCR product amplification, melting curves were measured. All samples were run in triplicates. Analysis was done using a CFX96^{TM} Real-Time Detection System (Bio-Rad, Vienna, Austria) and calculations were made according to the ΔΔCq-method [58] using Microsoft Excel (Office 2010).

For each gene of interest a calibration curve was established. The efficiency of all primer pairs was slightly below or above 100% (Bio-Rad CFX Manager 2.0).

Relative gene expression was assessed by normalization of expression levels to the transcriptional level of the housekeeping gene HPRT1.

Table 4 – Primer Sequences

NCBI RefSeq	Gene ID	Forward Primer Reverse Primer	Product Size
NM_004994	MMP9	TTGATCTTCCAAGGCCAATC CAGAAGCCCCACTTCTTGTC	290bp
NM_178812.3	MTDH	GGCAATTGGGTAGACGAAGA CCTGTTTTGGACGGGTTTTA	250bp
NM_000194.2	HPRT1	GACCAGTCAACAGGGGACAT CTGCATTGTTTTGCCAGTGT	111bp

4. RESULTS

4.1 Primary culture of WEAL

Within four weeks after thawing the frozen specimen and setting up the primary culture encoded "WEAL", an almost confluent monolayer had grown, giving rise to cells towering over another (Fig. 8 **A** and **B**). Collagenase treatment of the initial flask also yielded in adherently growing cells exhibiting an astral shape (Fig. 8 **C**).

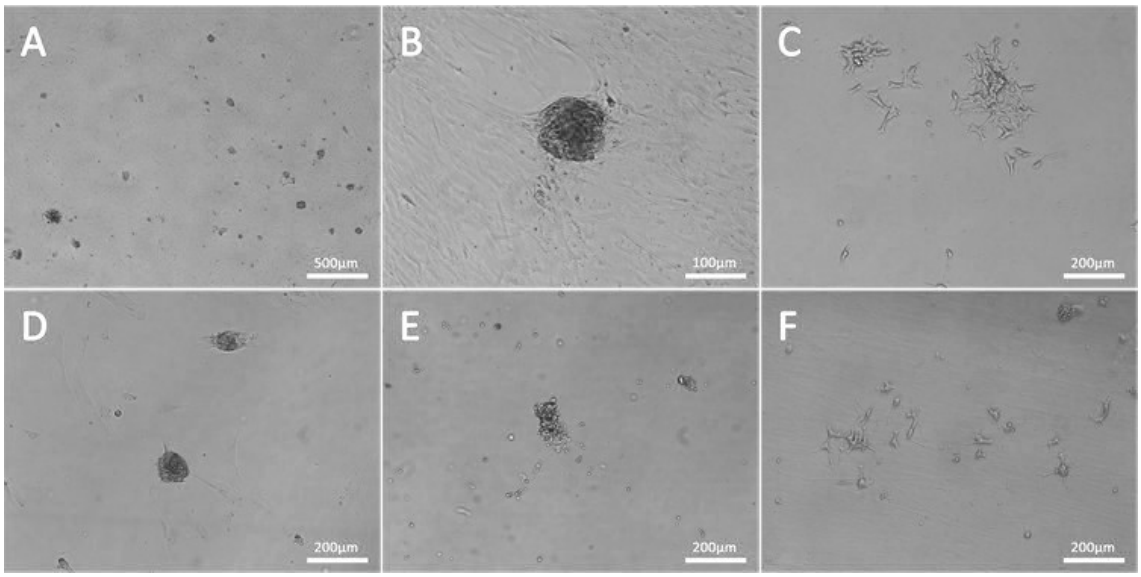


Fig. 8 – PRIMARY CULTURE OF WEAL. Upper row: Images taken after four weeks of cultivation. **A** Formation of a monolayer and arising "cell buds" (magnification: 40x). **B** Detailed view of a "cell bud" (magnification: 200x). **C** Adherently growing cells with astral shape (magnification: 100x). Lower row: Images taken after eight months of cultivation (magnification: 100x). **D** Cells stacking on top of each other even after months in culture. **E** Floating population of cells. **F** Adherently growing cells.

Ten months later, ~20 flasks had originated through typical cell cultivation procedures. In one part of them cells were still growing in buds (Fig. 8 **D**) and eventually detaching, whereas other flasks contained cells growing in suspension (Fig. 8 **E**). A small portion of cells maintained an astral shape (Fig. 8 **F**) but those were only found in flasks coated with poly-D-lysine.

4.2 Inhibition of cell proliferation

The effects of acetylshikonin (AS), β,β-dimethylacrylshikonin (DMAS), shikonin (Shik) and a petroleum ether extract of the dried roots of *Onosma paniculata* (EX) on medullary thyroid carcinoma cell lines MTC-SK, SHER-I and TT were examined by cell counting.

To MTC-SK cells, concentrations ranging from 0.1µM to 0.5µM (for AS, DMAS and Shik) and from 0.01 µg/ml to 0.2 µg/ml (for EX) were applied. Cell numbers were determined after 24, 48 and 72 hours and are presented as percentage of untreated cells ("control"). As shown in Fig. 9 cell counts decrease with increasing concentrations and with increasing incubation times. Application of DMSO as solvent control ("control+DMSO") did not result in any significant decrease or increase of cell counts.

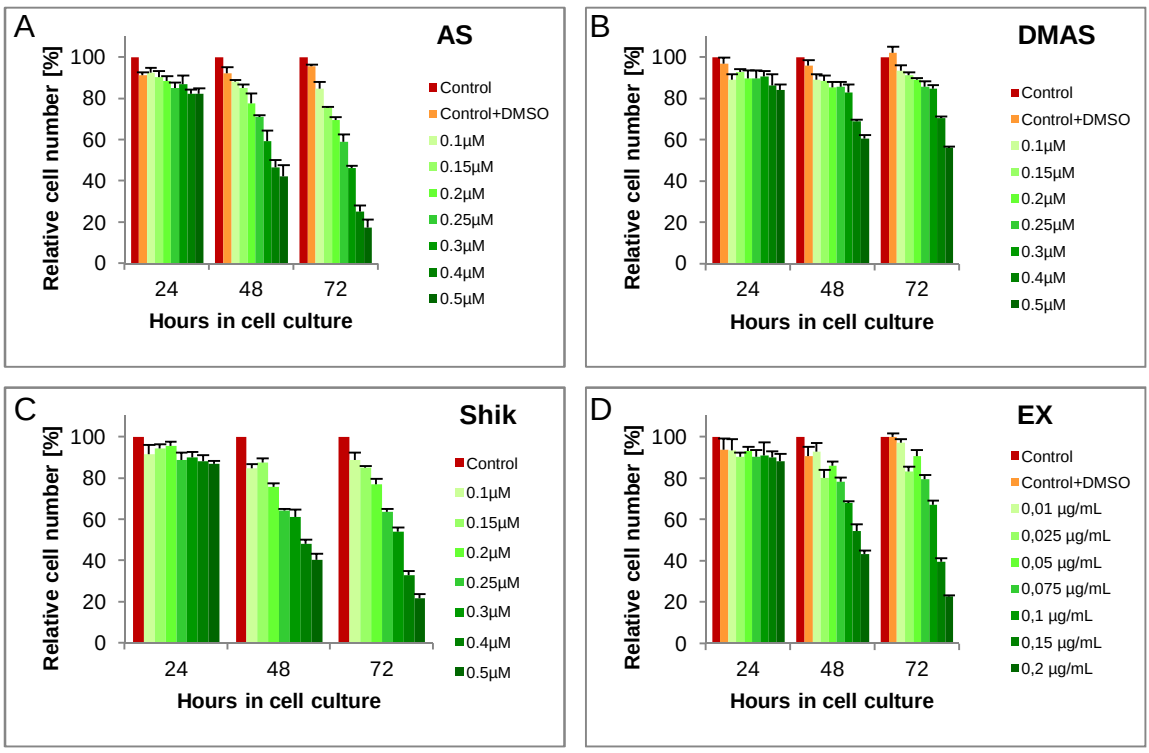


Fig. 9 – INHIBITORY EFFECTS OF SHIKONIN DERIVATIVES ON CELL PROLIFERATION OF MTC-SK. Cells were incubated with seven different concentrations ranging from 0.1µM to 0.5µM of acetylshikonin (**A**), dimethylacrylshikonin (**B**) and shikonin (**C**), and from 0.01 µg/ml to 0.2 µg/ml of petroleum ether extract (**D**). Cell numbers were determined after 24, 48 and 72 hours and are expressed as percentage of untreated control cells.

SHER-I cells were incubated with concentrations ranging from 0.01µM to 0.1µM of AS, 0.01µM to 0.15µM of DMAS, 0.05µM to 0.25µM of Shik or 0.01 µg/ml to 0.05 µg/ml of EX, respectively. Cell numbers were again measured after 24, 48 and 72 hours. Fig. 10 shows the decrease of cell numbers with increasing concentrations and incubation times. Results are presented as percentage of untreated control cells. Application of DMSO as solvent control did not show any significant effect on cell proliferation.

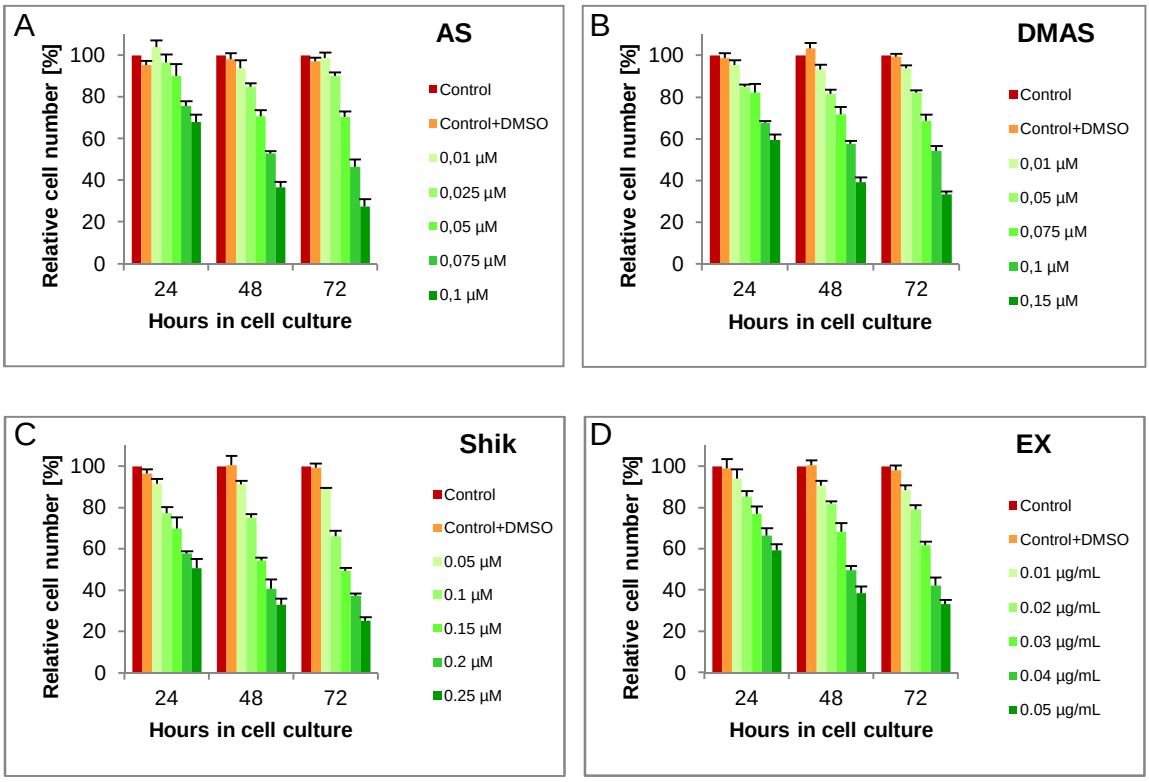


Fig. 10 – INHIBITORY EFFECTS OF SHIKONIN DERIVATIVES ON CELL PROLIFERATION OF SHER-I. Cells were incubated with various concentrations of **A** acetylshikonin, **B** dimethyl-acrylshikonin, **C** shikonin, **D** petroleum ether extract. Cell numbers were determined after 24, 48, and 72 hours and are presented as percentage of untreated control cells.

Hence, a time- and dose-dependent inhibitory effect on cell proliferation was observed with all tested compounds for suspension cell lines MTC-SK and SHER-I (Fig. 9 and 10).

Adherently growing TT cells also showed a dose-dependent reduction of cell proliferation when incubated with shikonin derivatives. Cells were incubated with concentrations ranging from 0.1µM to 2µM for AS, DMAS and Shik (Fig. 11 **A**) and from 0.05 µg/ml to 2 µg/ml for EX (Fig. 11 **B**), respectively. Cell numbers were determined after 72 hours of incubation and are presented as percentage of untreated control cells.

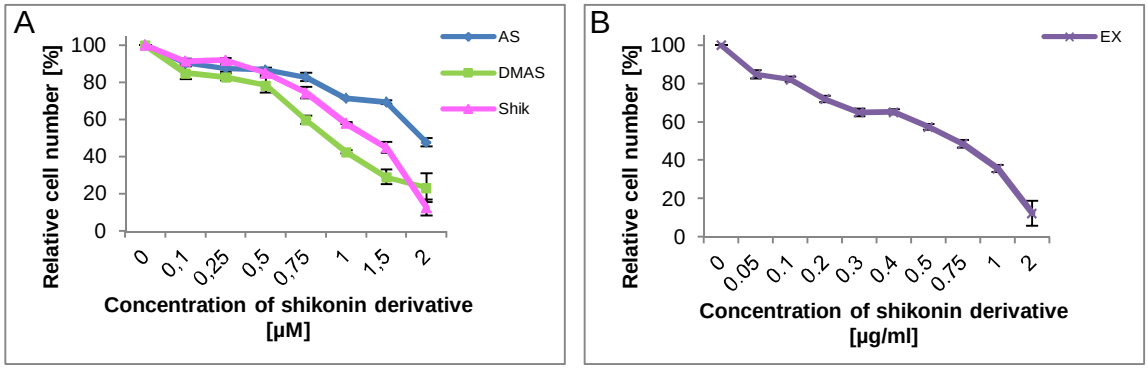


Fig. 11 – INHIBITORY EFFECTS OF SHIKONIN DERIVATIVES ON CELL PROLIFERATION OF TT. Cells were incubated with various concentrations of acetylshikonin, dimethylacrylshikonin, shikonin (**A**) and petroleum ether extract (**B**) for 72 hours.

Subsequently, IC_{50} values of the shikonin derivatives were calculated for each cell line. IC_{50} values, the inhibitory concentrations of the half-maximal effect, represent the concentrations required to reduce cell proliferation to 50%. Mean values ± SD of at least three independent experiments are presented in Table 5. Results show that IC_{50} values are ≤ 0.5 µM for suspension cells and range from approximately 1 to 2 µM for adherently growing cells. It should also be noted that IC_{50} values are lower after 72 hours of incubation than after 48 hours and that SHER-I cells are more susceptible to shikonin derivatives than MTC-SK cells.

Table 5 – IC_{50} values of shikonin derivatives for MTC cell lines

	TT	MTC-SK		SHER-I	
Compound	**72h**	**48h**	**72h**	**48h**	**72h**
AS [µM]	2.1 ± 0.4	0.39 ± 0.04	0.32 ± 0.04	0.09 ± 0.02	0.09 ± 0.02
DMAS [µM]	1.2 ± 0.3	0.50 ± 0.15	0.49 ± 0.13	0.14 ± 0.02	0.13 ± 0.02
Shik [µM]	1.1 ± 0.2	0.31 ± 0.08	0.30 ± 0.02	0.18 ± 0.01	0.17 ± 0.01
EX [µg/ml]	0.6 ± 0.2	0.16 ± 0.04	0.14 ± 0.03	0.05 ± 0.01	0.04 ± 0.01

In order to be able to compare the values of the pure substances acetylshikonin, dimethylacrylshikonin and shikonin to the unfractionated petroleum ether extract, IC_{50} concentrations were converted to µg/ml according to their molecular weight. As shown in Fig. 12, treatment with the extract, which is a mixture of shikonin derivatives, did not result in lower IC_{50} values than application of the pure compounds. On the contrary, acetylshikonin and shikonin showed the most effect on suspension cells MTC-SK and SHER-I, whereas shikonin and dimethylacrylshikonin had the most impact on adherent cells TT.

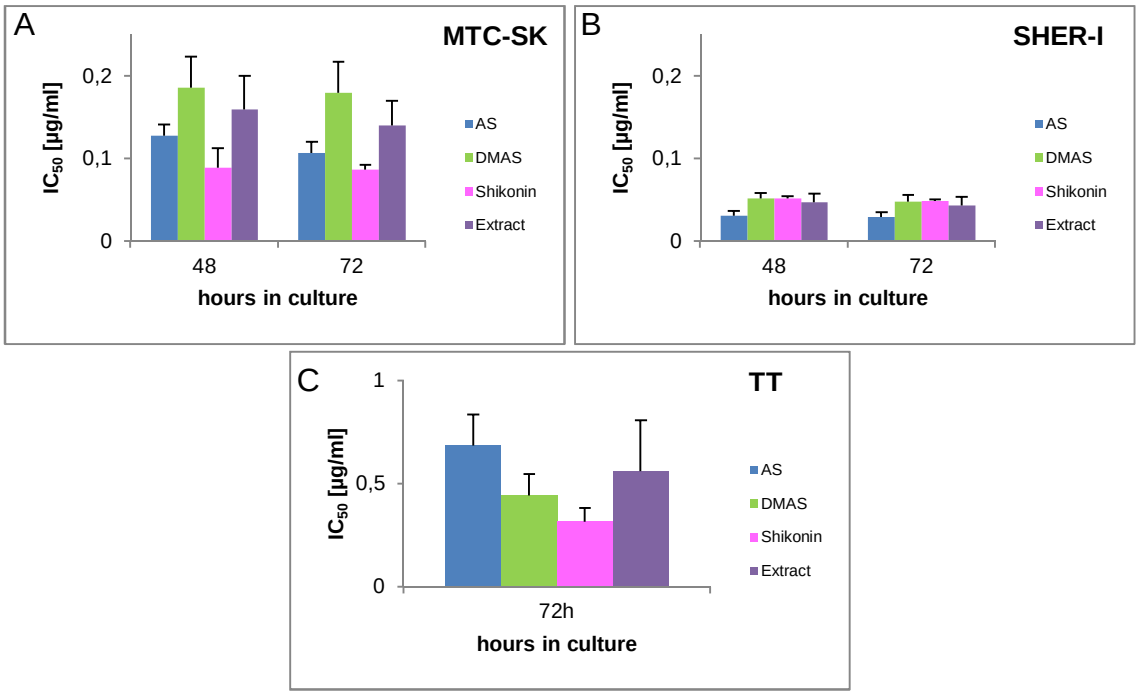


Fig. 12 – COMPARISON OF IC_{50} VALUES AFTER CONVERSION FROM [µM] TO [µg/ml].
A MTC-SK cells are most susceptible to acetylshikonin and shikonin. **B** Acetylshikonin shows the highest potency towards SHER-I cells. **C** Shikonin has the biggest effect on TT cells.

4.3 Reduction of cell viability

Treatment with shikonin derivatives also reduced cell viability of MTC cell lines in a time- and dose-dependent manner. Fig. 13 shows a representative plot of SHER-I cells being incubated with seven different concentrations ranging from 0.05µM to 0.75µM for AS, DMAS and Shik, and from 0.01 µg/ml to 0.2 µg/ml for EX. Cell viability was determined after 24, 48 and 72 hours of incubation. Results were normalized to untreated control cells ("control").

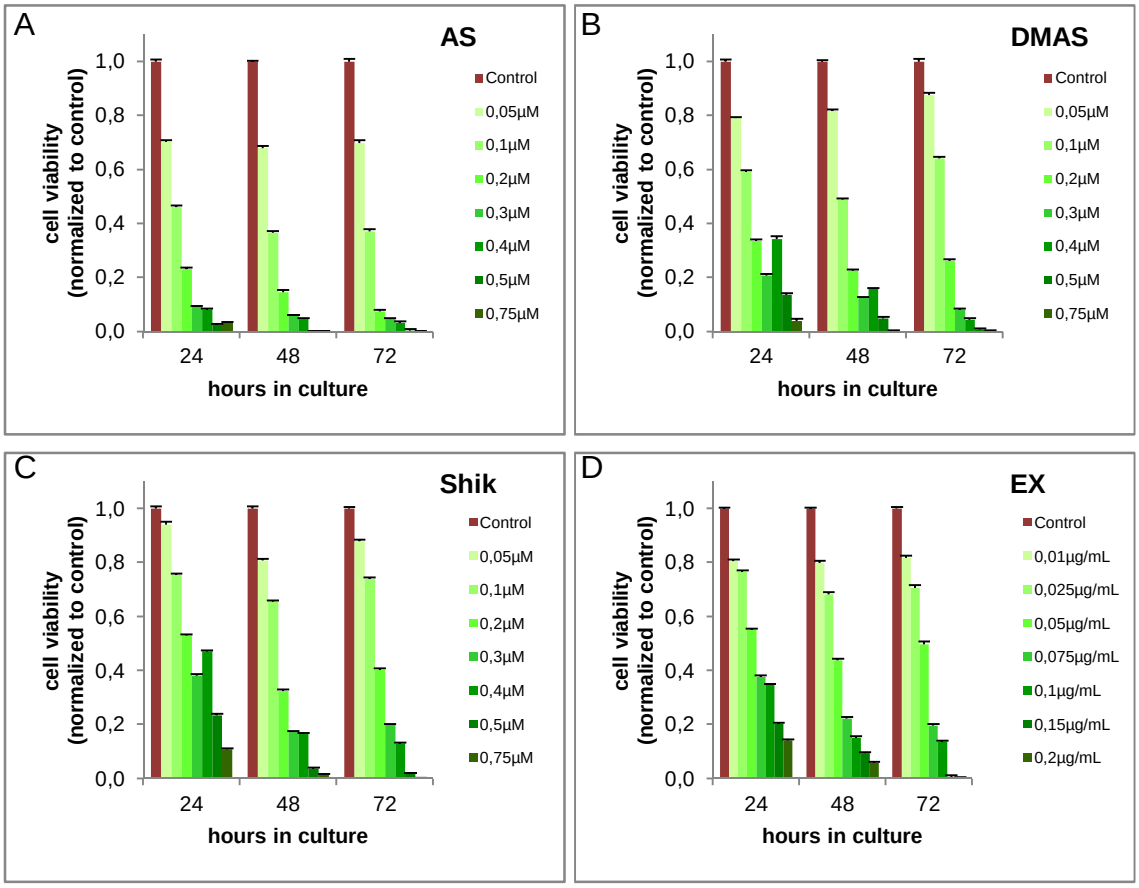


Fig. 13 – REDUCTION OF CELL VIABILITY. Representative plots for the reduction of cell viability after the application of various concentrations of acetylshikonin (**A**), dimethylacrylshikonin (**B**), shikonin (**C**) and petroleum ether extract (**D**) on SHER-I cells. Cell viability was determined after 24, 48 and 72 hours of incubation. Results were normalized to untreated control cells.

Normal human skin fibroblasts, HF-SAR, on the contrary, showed no reduction of cell viability when incubated with the IC_{50} concentrations calculated for MTC-SK cells (Fig. 14 **A**), and only a slight reduction of cell viability when incubated with the IC_{50} concentrations calculated for TT cells (Fig. 14 **B**).

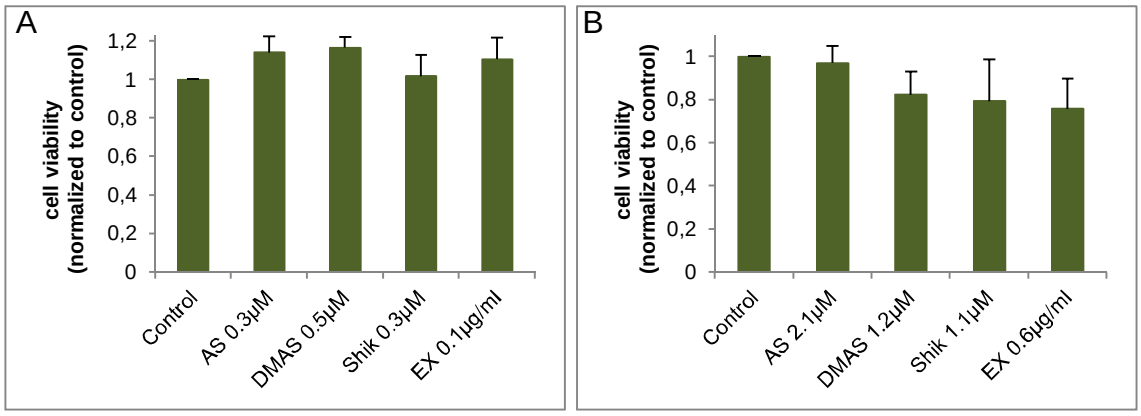


Fig. 14 – CELL VIABILITY OF NORMAL HUMAN SKIN FIBROBLASTS. HF-SAR cells do not show any reduction in cell viability when incubated with the IC_{50} concentrations of shikonin derivatives calculated for MTC-SK cells (**A**) and only a slight reduction with those for TT cells (**B**). Cell viability was determined after 72 hours of incubation with the indicated concentrations. Results were normalized to untreated control cells.

4.4 Morphological changes

MTC-SK and SHER-I cells normally grow in suspensions of single cells and multicellular aggregates (Fig. 15 **A**,**B** and Fig. 16 **A**-**C**). Incubation with the corresponding IC_{50} concentrations of shikonin derivatives inhibited aggregate formation of MTC-SK cells (Fig. 15 **C**-**J**) and strongly reduced it with SHER-I cells (Fig. 16 **D**-**O**).

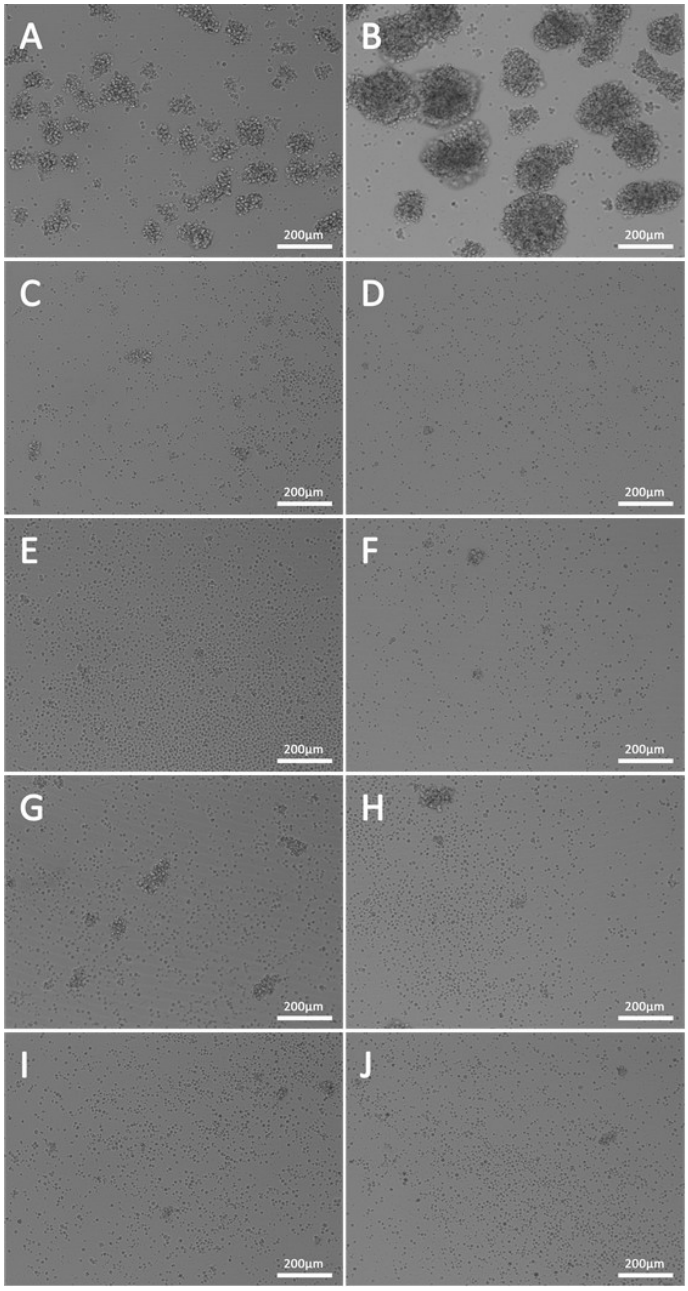


Fig. 15 – SHIKONIN DERIVATIVES INHIBIT AGGREGATE FORMATION OF MTC-SK CELLS. Cells were pipetted into single cells and incubated with DMSO as solvent control (**A**,**B**) or the corresponding IC_{50} values of acetylshikonin (**C**,**D**), dimethylacrylshikonin (**E**,**F**), shikonin (**G**,**H**) and petroleum ether extract (**I**,**J**) for 24 hours (left column) and 48 hours (right column).

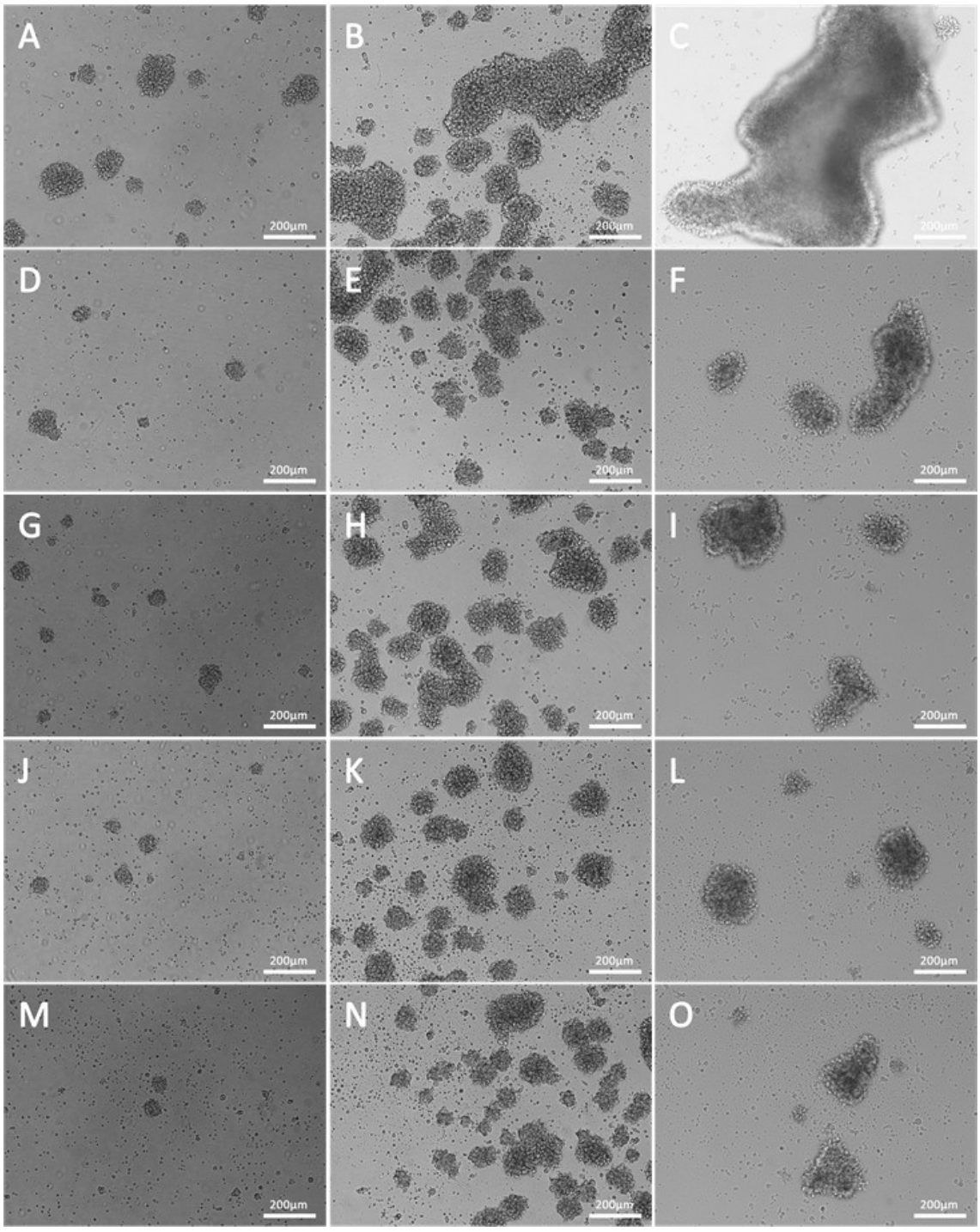


Fig. 16 – SHIKONIN DERIVATIVES REDUCE AGGREGATE FORMATION OF SHER-I CELLS. Cells were pipetted into single cells and incubated with DMSO as solvent control (**A**-**C**) or the corresponding IC_{50} values of the shikonin derivatives acetylshikonin (**D**-**F**), dimethylacrylshikonin (**G**-**I**), shikonin (**J**-**L**) and the petroleum ether extract (**M**-**O**) for 24 hours (left column), 48 hours (middle column) and 72 hours (right column).

Morphological changes were also observed in adherently growing TT cells after the application of shikonin derivatives. Cells showed a round rather than a polygonal shape and detached from cell culture flasks. Fig. 17 shows images of TT cells treated with DMSO as solvent control (**A**,**B**) and representative images after incubation with the IC_{50} concentration of shikonin derivatives (**C**,**D**).

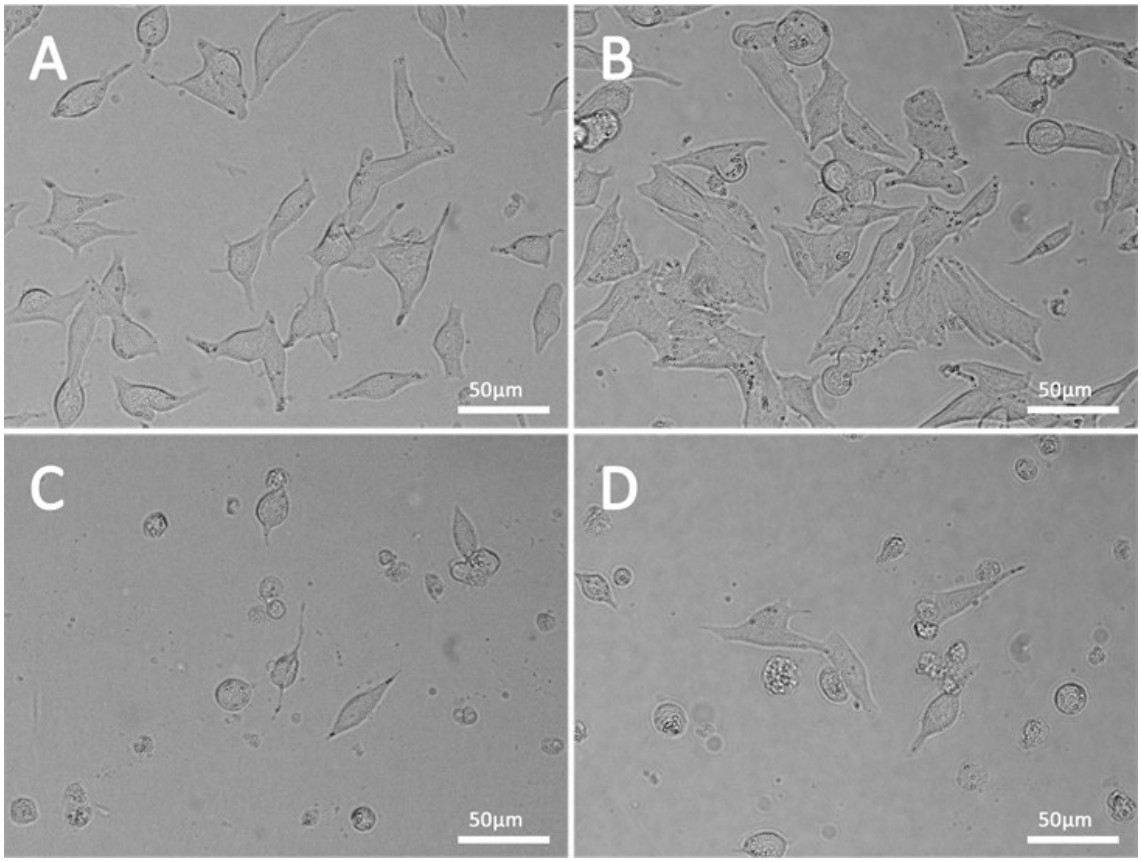


Fig. 17 – MORPHOLOGICAL CHANGES OF TT CELLS UPON INCUBATION WITH SHIKONIN DERIVATIVES. Cells were seeded and allowed to attach overnight before they were incubated with DMSO (**A**, **B**) and the corresponding IC_{50} concentration of shikonin (**C**, **D**) for 24 hours (left column) and 48 hours (right column).

4.5 Nucleic alterations

Staining with the fluorescent Hoechst dye allows the visualization of nuclei of live cells. Untreated control cells exhibited nuclei of round shape that engross almost the entire cell (Fig. 18 **A**, **B** and Fig. 19 **A**, **B**), whereas treatment of MTC cells with the IC_{50} values of shikonin derivatives resulted in smaller, more compact nuclei (Fig. 18, **C**-**F** and Fig. 19 **C**-**F**).

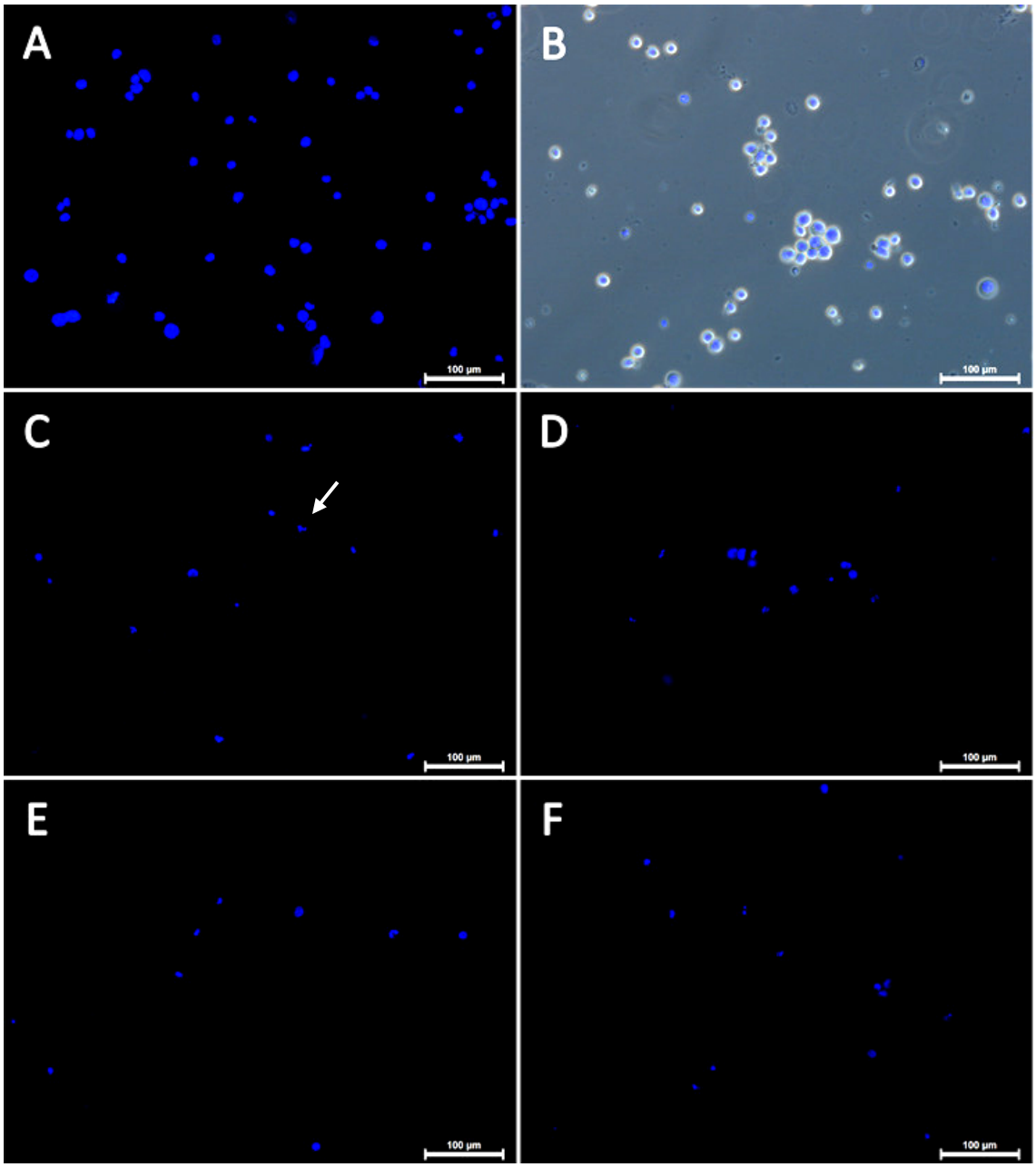


Fig. 18 – FLUORESCENCE MICROSCOPY OF HOECHST STAINED MTC-SK CELLS. A Untreated control cells. **B** Overlay of fluorescence and phase-contrast images of untreated control cells. Cells exhibit round nuclei, whereas treatment with acetylshikonin (**C**), dimethylacrylshikonin (**D**), shikonin (**E**) and the petroleum ether extract (**F**) equivalent to their IC_{50} concentrations lead to fragmented nuclei. One example thereof is indicated by the arrow in **C**.

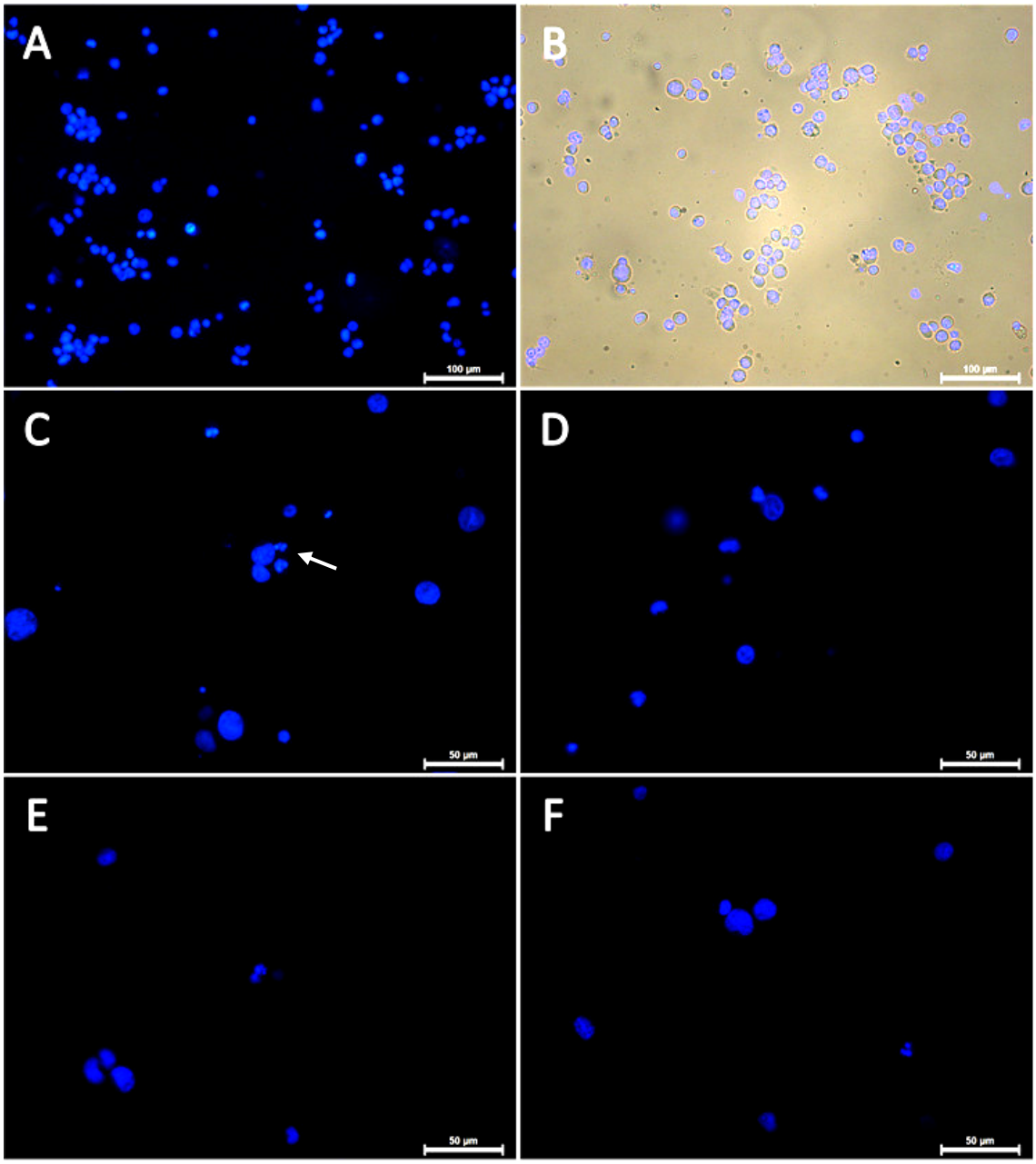


Fig. 19 – FLUORESCENCE MICROSCOPY OF HOECHST STAINED SHER-I CELLS.
A Untreated control cells. **B** Overlay of fluorescence and phase-contrast images of untreated control cells. Chromatin condensation and apoptotic bodies (as indicated by the arrow) were detected after treatment with the corresponding IC_{50} values of acetylshikonin (**C**), dimethylacrylshikonin (**D**), shikonin (**E**) and the petroleum ether extract (**F**).

Characteristic traits of apoptosis like cell shrinkage, chromatin condensation (Fig. 20), nucleic fragmentation and the formation of apoptotic bodies were detected in MTC-SK and SHER-I cells after 24 hours of treatment with shikonin derivatives.

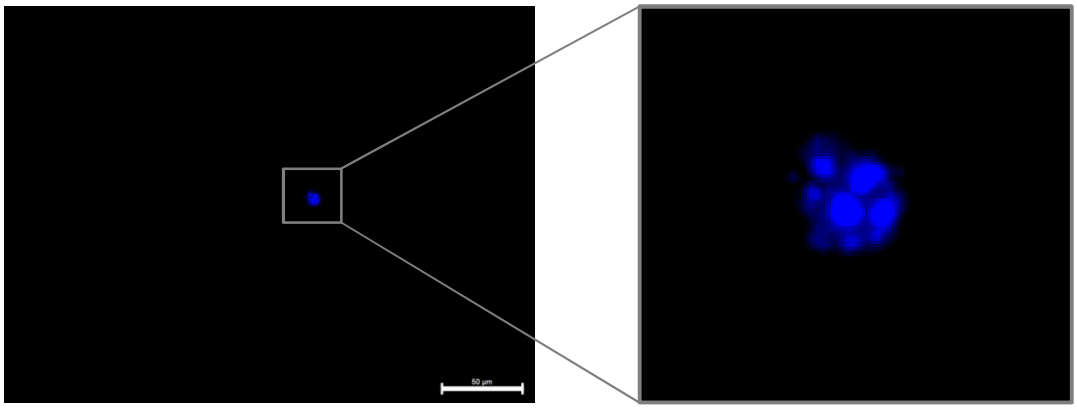


Fig. 20 – CHROMATIN CONDENSATION OF MTC-SK CELL NUCLEUS. Fluorescence image of one single MTC-SK cell nucleus exhibiting fragmentation. The image was acquired after incubation with 0.3µM (IC_{50}) acetylshikonin for 24 hours.

4.6 Activation of apoptotic pathways

Incubation of MTC-SK and SHER-I cells with shikonin derivatives led to the up-regulation of effector caspases 3 and 7 (Fig. 21).

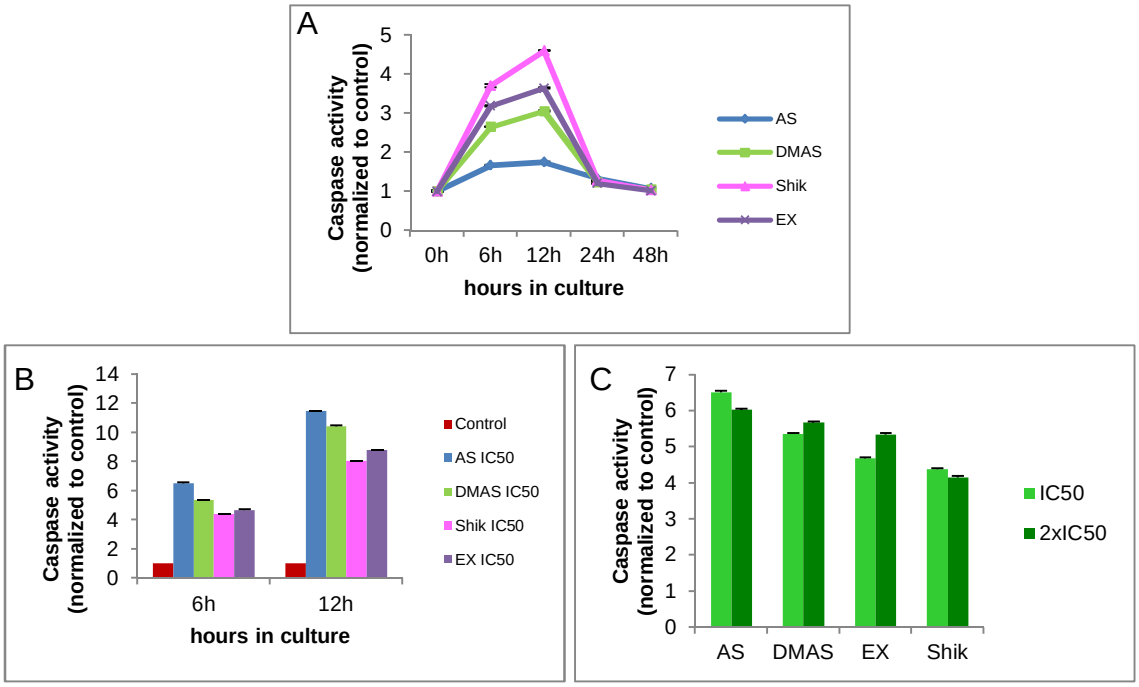


Fig. 21 – UP-REGULATION OF CASPASE 3 AND 7. A Effector caspases are up-regulated in SHER-I cells after 6 and 12 hours of treatment with shikonin derivative concentrations according to the IC_{50} values, but not anymore after 24 or 48 hours. **B** Also in MTC-SK cells the apoptotic pathway is activated after application of the corresponding IC_{50} concentrations. **C** However, caspases are not activated in a dose-dependent way after 6 hours of treatment in MTC-SK.

The apoptotic pathway was found to be activated after 6 and 12 hours of treatment with the substances, but not anymore after 24 or 48 hours. Interestingly, increasing the concentration from IC_{50} to $2xIC_{50}$ did not enhance caspase activity. Apparently, effector caspases 3 and 7 were activated in a time-, but not a dose-dependent way.

Another hallmark of the induction of apoptosis is the "flipping" of phosphatidylserine (PS) from the cytosolic side of the membrane to the cell surface. Fluorescence labeled Annexin V binds to PS and thus allows the visualization of apoptotic effects by flow cytometry.

Placement of the gate was performed in a way that both live and dead cells were analyzed, but not cell debris (Fig. 22 **A**, **B**). Plotting of the two measured fluorophores, i.e. FITC-labeled Annexin V and 7-AAD, against each other enables the distinction between live cells (lower left quadrant), early and late apoptotic (lower and upper right quadrant, respectively), and dead cells (upper left quadrant). Fig. 23 **A**-**C** show representative plots of the induction of apoptosis by acetylshikonin in MTC-SK cells.

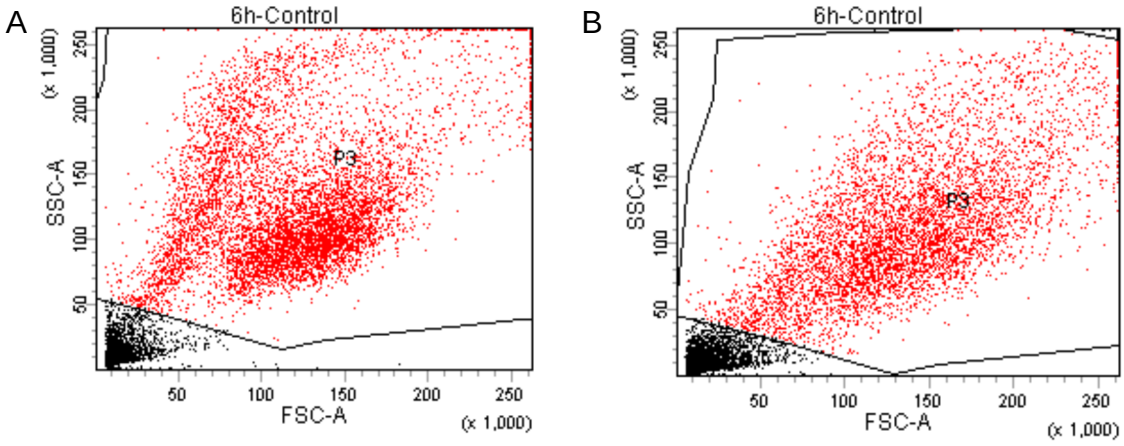


Fig. 22 – FLOW CYTOMETRY OF MTC CELL LINES. Gating of MTC-SK (**A**) and SHER-I (**B**). The gates were chosen in a way that they included live, apoptotic and dead cells, but not cell debris.

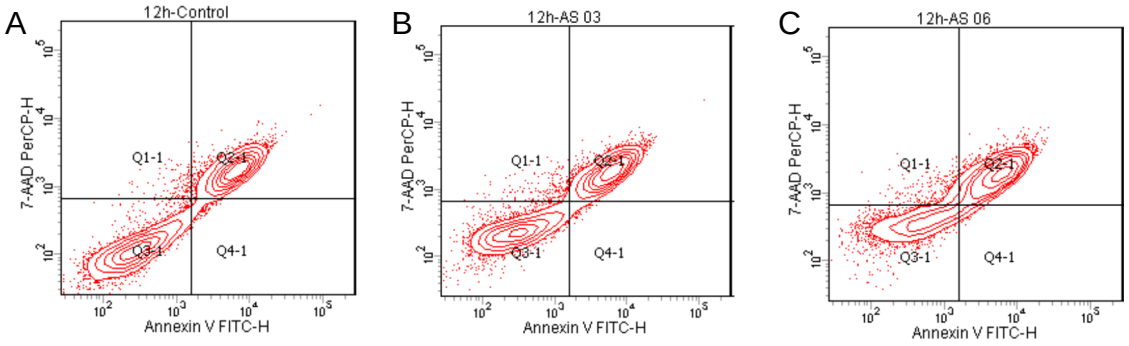


Fig. 23 – DISTINCTION BETWEEN LIVE AND APOPTOTIC CELLS BY FLOW CYTOMETRY. MTC-SK cells were incubated with DMSO as solvent control (**A**), 0.3µM (IC_{50}) acetylshikonin (**B**) and 0.6µM ($2xIC_{50}$) acetylshikonin (**C**) for 12 hours. A reduction in live cells (lower left quadrant) and an increase in apoptotic cells (upper right quadrant) were detected.

For a more detailed analysis of time- and dose-dependent effects of shikonin derivatives on MTC cell lines, Annexin V fluorescence of the gated population was plotted against the amount of counts. The results show that apoptosis was induced in MTC-SK (Fig. 24) and SHER-I (Fig. 25) by acetylshikonin and shikonin in a time- and dose-dependent way.

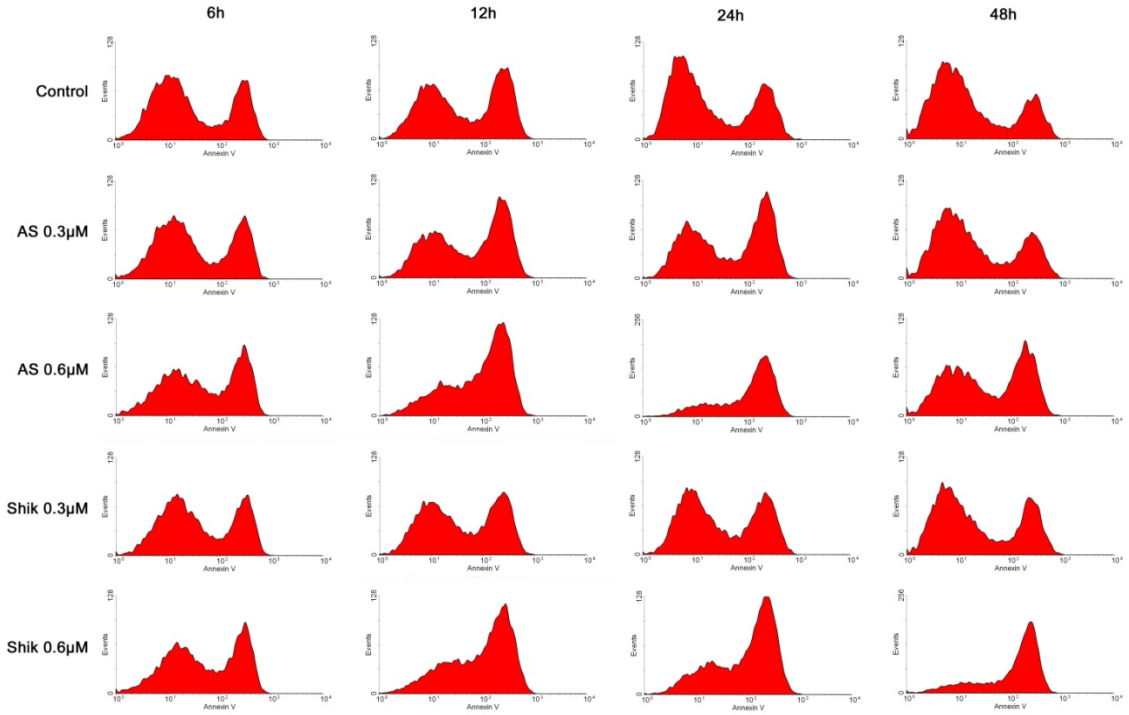


Fig. 24 – ANNEXIN V BINDING OF MTC-SK. Cells were incubated with DMSO as solvent control or the corresponding IC_{50} and $2xIC_{50}$ concentrations as indicated for 6, 12, 24 and 48 hours, respectively.

Incubation of MTC-SK cells with the concentrations of acetylshikonin and shikonin equivalent to their IC_{50} and $2xIC_{50}$ values for 6 hours only showed a slight effect on Annexin V binding. However, after 12 hours of treatment, the fluorescence intensity of healthy cells decreased and the fluorescence levels of apoptotic cells increased remarkably. This effect was even enhanced 24 hours after the application of the compounds, especially when the $2xIC_{50}$ concentrations were applied. After 48 hours, the proportion of apoptotic cells decreased again.

In SHER-I cells, incubation with acetylshikonin and shikonin for 6 hours at their respective IC_{50} and $2xIC_{50}$ concentrations also only showed a slight effect, whereas incubation for 12 hours drastically decreased the amount of live cells (see Fig. 25). This is reflected by the reduction of the fluorescence of healthy cells (left peak) and the concomitant increase of the fluorescence of apoptotic cells (right peak). As opposed to MTC-SK, incubation of SHER-I for 24 hours did not result in higher rates of apoptotic cells.

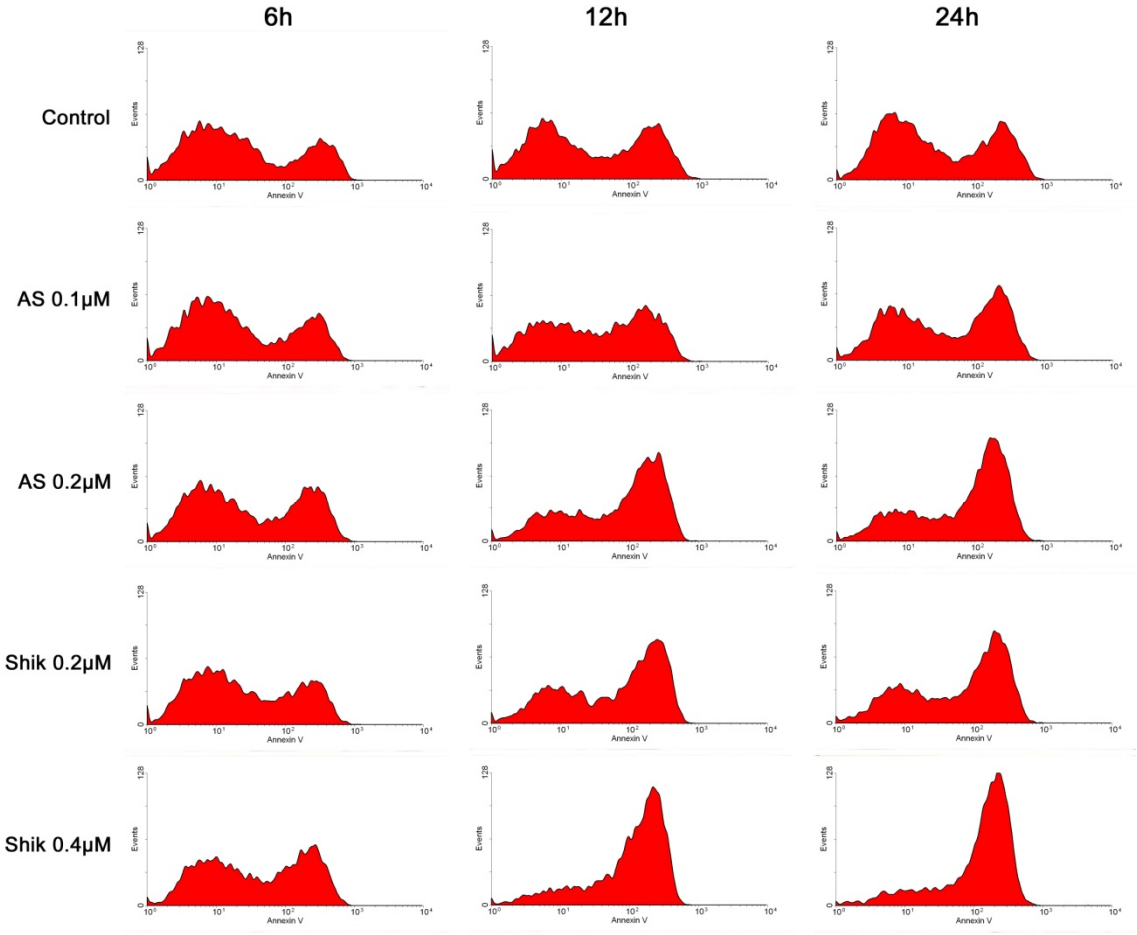


Fig. 25 – ANNEXIN V BINDING OF SHER-I. Cells were incubated with DMSO as solvent control or the corresponding IC_{50} and 2xIC_{50} concentrations as indicated for 6, 12 and 24 hours, respectively. Application of shikonin derivatives decreases the amount of live cells and increases the amount of apoptotic cells.

4.7 Breakdown of the mitochondrial transmembrane potential

The mitochondrial transmembrane potential (ΔΨm) is essential for the generation of ATP via oxidative phosphorylation. Impairment of the proton gradient generated by the electron transport chain leads to the release of cytochrome c and consequently the activation of caspases [59].

Alteration of ΔΨm can be assessed by JC-1 staining. In intact mitochondria JC-1 forms aggregates that exhibit a red fluorescence, whereas depolarization of the mitochondrial membrane leaves JC-1 in a monomeric state with green fluorescence.

The effects of shikonin and acetylshikonin on ΔΨm of suspension cell lines MTC-SK and SHER-I were studied through flow cytometry. For MTC-SK the gate was chosen in a way that it excluded dead cells and cell debris of the control sample (Fig. 26 **A**). These settings were then used for the analysis of treated cells (Fig. 26 B).

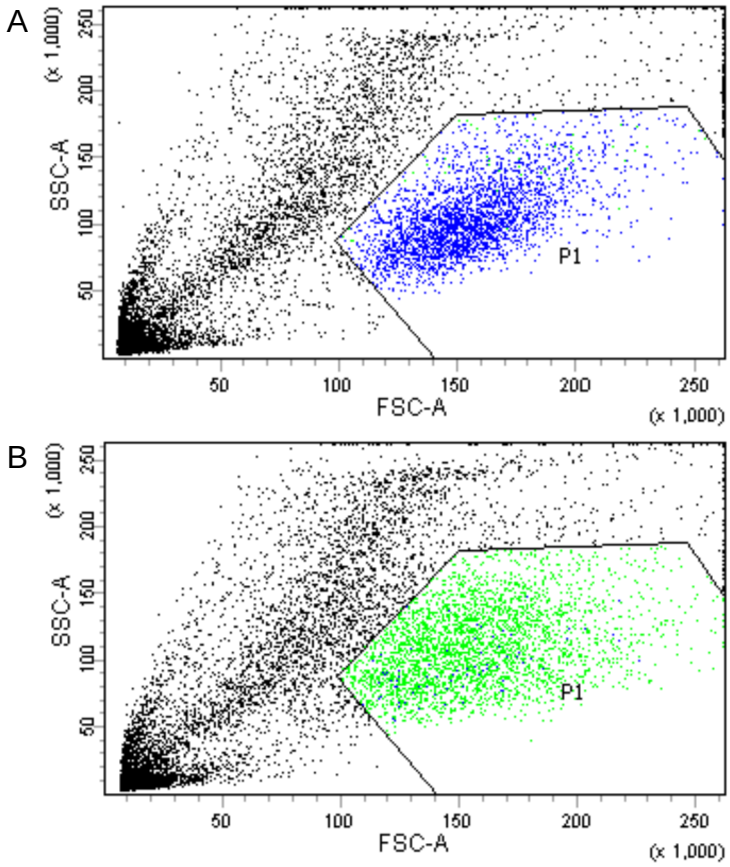


Fig. 26 – GATING IN JC-1 FLOW CYTOMETRY. A The gate includes live MTC-SK cells of the control sample only. **B** Analysis of treated cells was performed only on cells within the previously established gate.

MTC-SK cells were stained with JC-1 and treated with either DMSO as solvent control or 0.3µM (IC_{50}), 0.6µM ($2xIC_{50}$) and 1.2µM ($4xIC_{50}$) shikonin or acetylshikonin for 6 hours. Subsequently, the cellular shikonin signal [38] and both the red and green fluorescence of JC-1 were analyzed. Fig. 27 shows the results of MTC-SK cells upon incubation with the various acetylshikonin concentrations. Increasing concentrations led to a decrease of the red JC-1 fluorescence indicating a breakdown of the mitochondrial transmembrane potential (shown counterclockwise starting from the lower left plot).

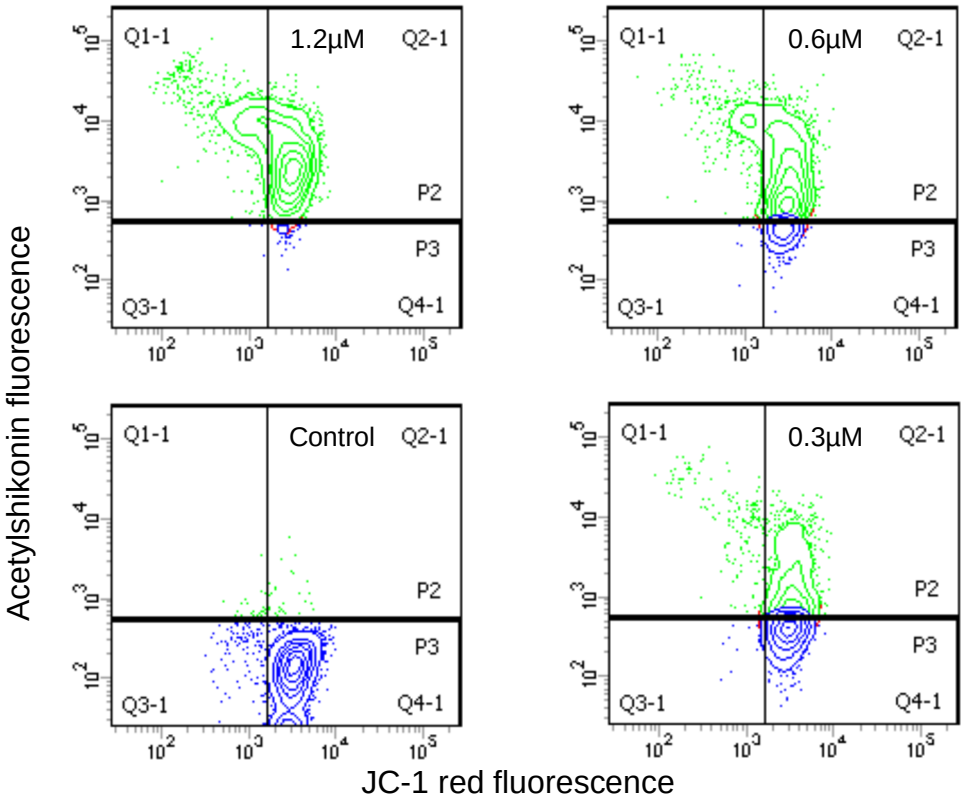


Fig. 27 – BREAKDOWN OF THE MITOCHONDRIAL TRANSMEMBRANE POTENTIAL. Acetylshikonin induced a dose-dependent reduction of the red JC-1 fluorescence in MTC-SK cells indicating the breakdown of ΔΨm.

For SHER-I cells, unfortunately, the experiment was infeasible. As previously shown (Fig. 22 **B**), gating of the control cells is sophisticated since there is no clear margin between live and dead cells. Hence, the exclusion of JC-1 fluorescence originating from "naturally" dead cells cannot be excluded. Resulting plots are indecisive (data not shown).

According to the manufacturer's protocol, a decrease in red fluorescence is in accordance with an increase in green fluorescence as JC-1 remains in its monomeric state in depolarized mitochondria. In the conducted experiments, however, both the red and the green fluorescence decreased with increasing concentrations of shikonin derivatives (Fig. 28).

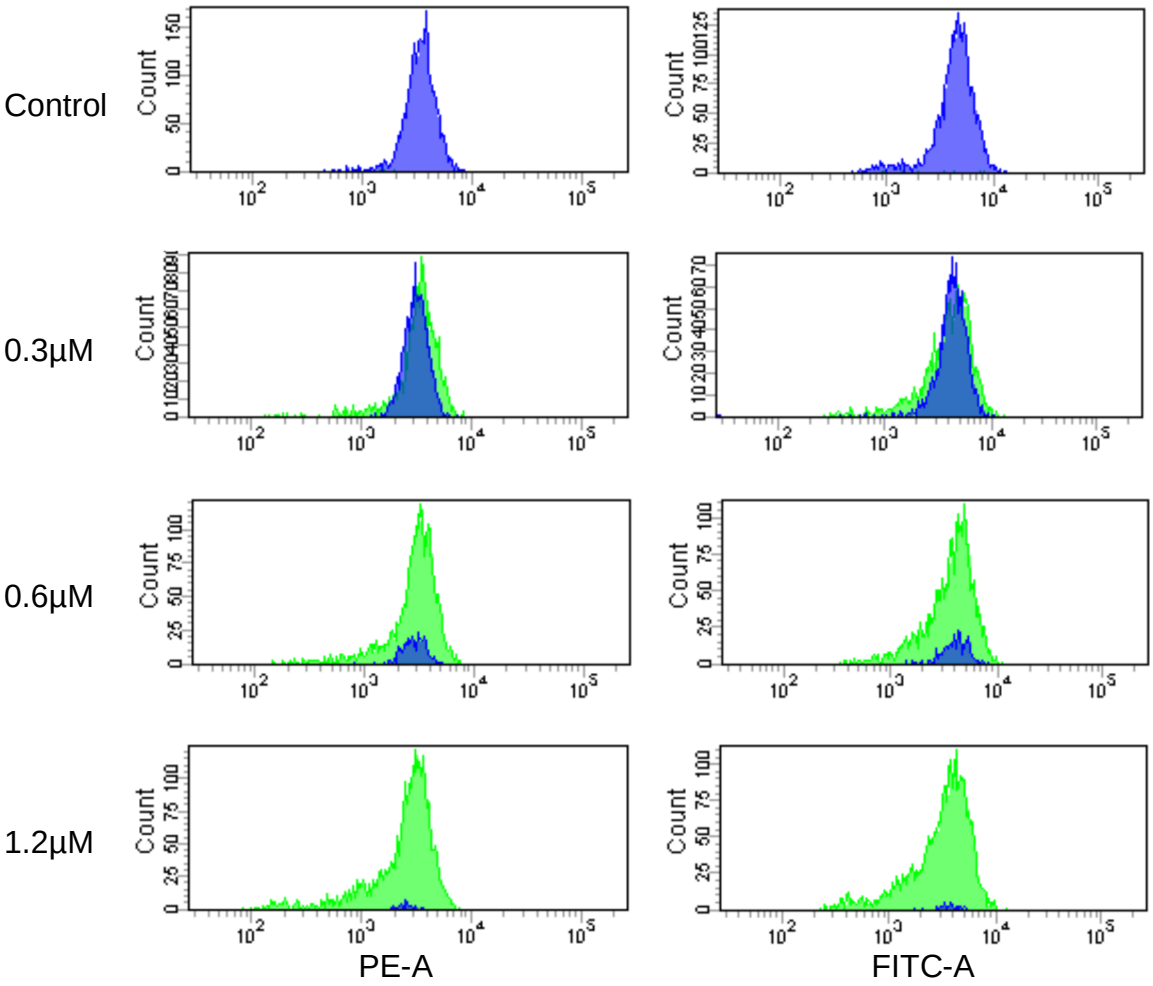


Fig. 28 – FLOW CYTOMETRY OF JC-1 STAINED MTC-SK CELLS. Incubation with various concentrations of acetylshikonin led to a decrease in both the red and the green fluorescence of JC-1 indicating artifacts.

4.8 Cell cycle arrest

Changes in cell cycle distribution were studied in MTC-SK and SHER-I cells after 24 hours of incubation with concentrations of acetylshikonin and shikonin equivalent to IC_{50} and $2xIC_{50}$. With increasing concentrations of the respective compound, the percentage of cells in G2/M decreased and the percentage of cells in G0/G1 increased. This cell cycle arrest also resulted in increasing sub-G0 peaks which again indicates the induction of apoptosis. Representative plots of two independent experiments are shown in Fig. 29 and Fig. 31. For numeric analysis thereof see Fig. 30.

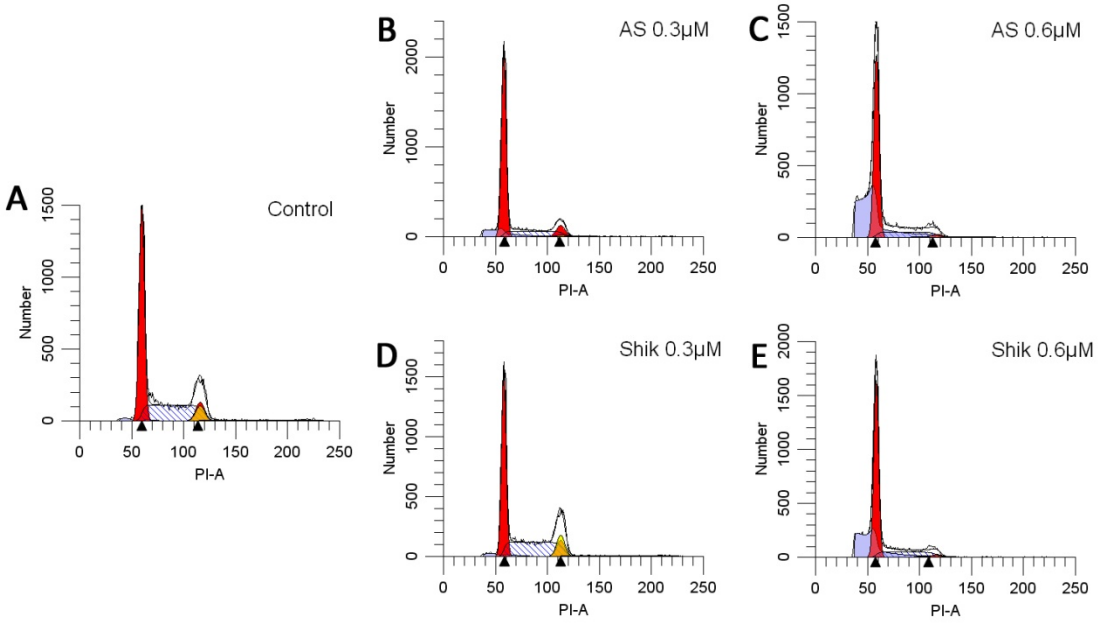


Fig. 29 – CELL CYCLE ANALYSIS OF MTC-SK. Cells were incubated with DMSO as solvent control (**A**), 0.3µM (IC_{50}) acetylshikonin (**B**), 0.6µM ($2xIC_{50}$) acetylshikonin (**C**), 0.3µM (IC_{50}) shikonin (**D**) and 0.6µM ($2xIC_{50}$) shikonin (**E**). Application of shikonin derivatives leads to the formation of a sub-G0 peak.

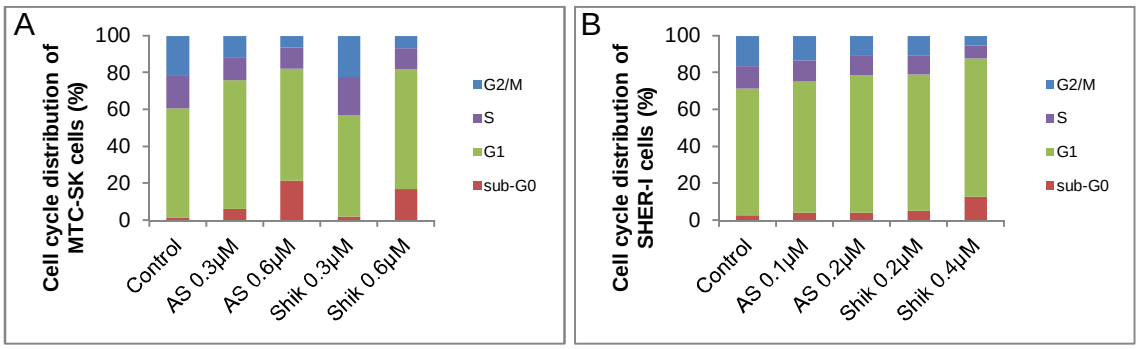


Fig. 30 – CELL CYCLE QUANTIFICATION. Acetylshikonin and shikonin induce a cell cycle arrest in MTC-SK (**A**) and SHER-I (**B**). Cells are arrested in G0/G1 which leads to a reduction of cells in G2/M and S-phase.

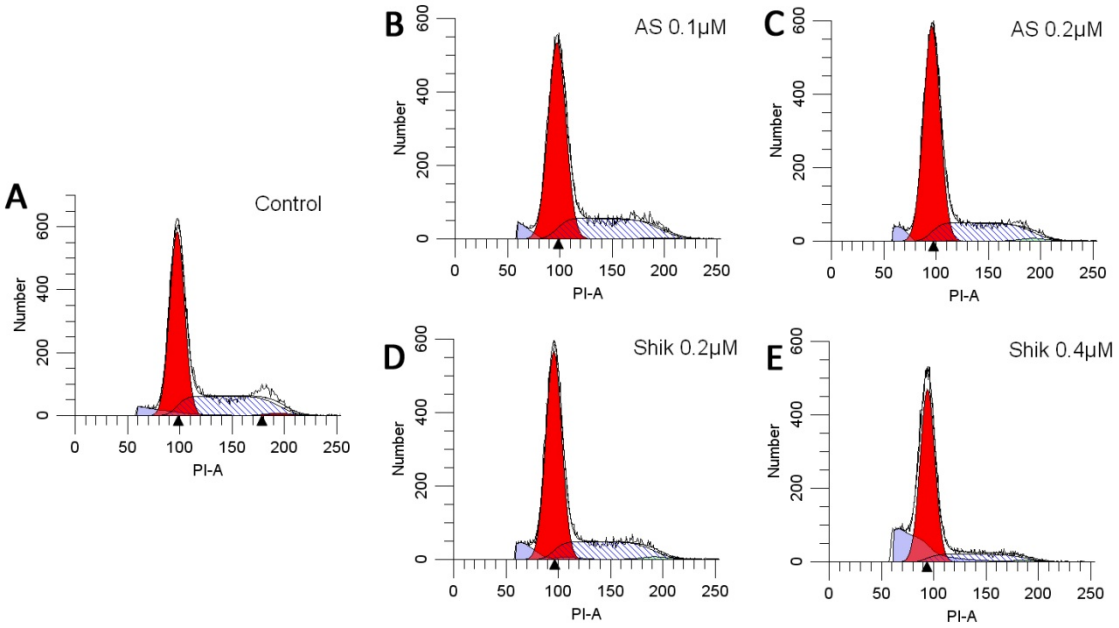


Fig. 31 – CELL CYCLE ANALYSIS OF SHER-I. Cells were incubated with DMSO as solvent control (**A**), 0.1µM (IC_{50}) acetylshikonin (**B**), 0.2µM ($2xIC_{50}$) acetylshikonin (**C**), 0.2µM (IC_{50}) shikonin (**D**) and 0.4µM ($2xIC_{50}$) shikonin (**E**). With increasing concentration of the shikonin compound the sub-G0 peak increases, whereas the amount of cells in G2/M and S-phase are decreased.

4.9 Inhibition of cancer cell migration

Cancer cell migration can be assayed by creating a scratch on a monolayer of adherent cells. Cells on the edges of the scratch will move towards the opening to close the gap and reestablish cell-cell contacts. Addition of shikonin derivatives to the cell culture medium at non-toxic concentrations (IC_{50}/5) inhibited cell migration significantly. Fig. 32 shows representative images acquired from a sample of untreated TT cells compared to TT cells treated with shikonin.

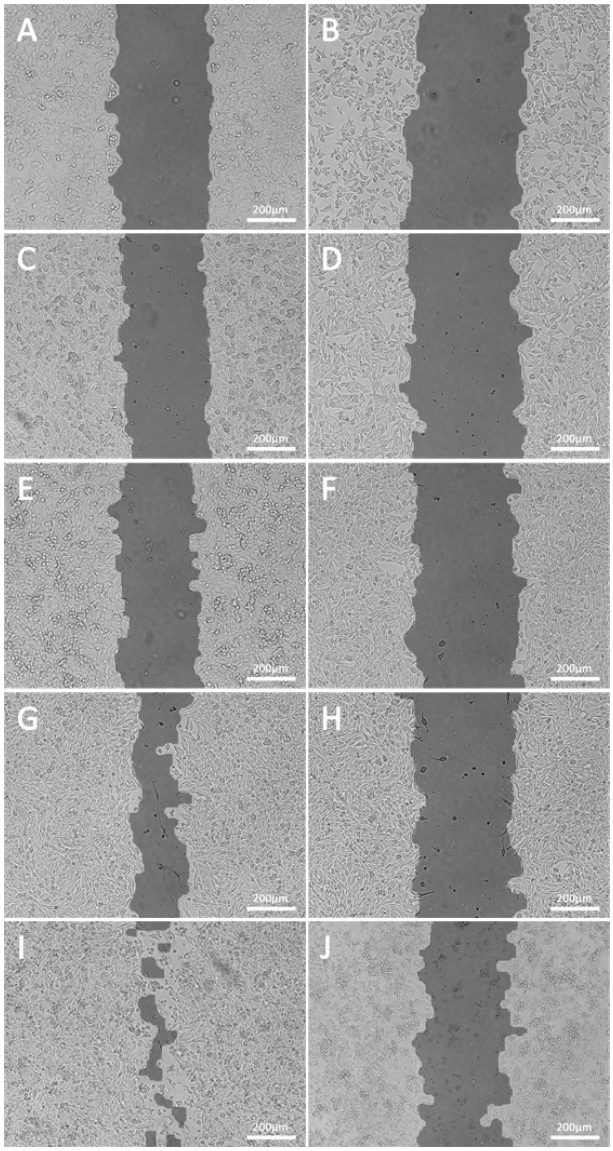


Fig. 32 – SHIKONIN DERIVATIVES INHIBIT CANCER CELL MIGRATION. TT cells were incubated with DMSO as solvent control (left column) or shikonin derivatives at concentrations equivalent to IC_{50}/5. Shown here are the results for treatment with shikonin (right column). Images were acquired immediately after the scratch was created (**A**,**B**) and 3 days (**C**,**D**), 5 days (**E**,**F**), 7 days (**G**,**H**) and 9 days (**I**,**J**) after that.

Images were analyzed with TScratch software (Fig. 33) and statistical analysis of three independent results was performed using a two-tailed unpaired student's t-test where $p<0.05$ was considered significant and $p<0.01$ very significant. Wound closure was calculated using the following formula:

$$\text{wound closure} = 100\% - \text{open wound area}$$

where

$$\text{open wound area} = \frac{\text{open image area day x}}{\text{open image area day 0}} \times 100\%$$

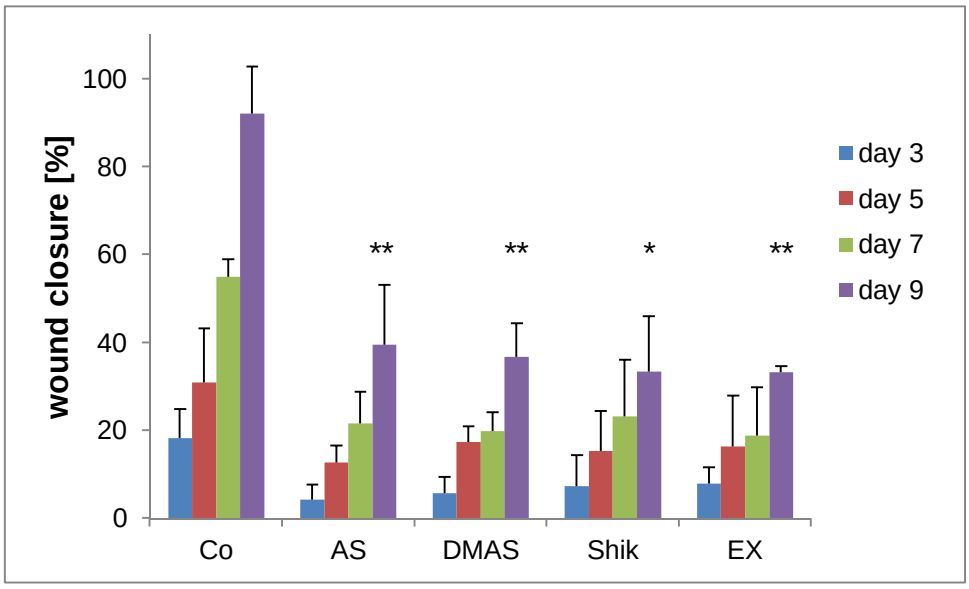


Fig. 33 – INHIBITION OF CANCER CELL MIGRATION BY SHIKONIN DERIVATIVES. Application of the corresponding $IC_{50}/5$ concentrations of shikonin derivatives inhibited migration of TT cells significantly (*; shikonin) and very significantly (**; acetylshikonin, dimethylacrylshikonin and petroleum ether extract).

It should be noted that full closure of the wound – even under optimized conditions – was only achieved 9 days after the scratch was created on a confluent monolayer of TT cells. Usual durations for wound closure in monolayer wound healing assays vary from 8 to 24 hours, according to literature [52]. However, the assay was performed nevertheless, since video evidence exists (unpublished data; courtesy of R. Fuchs) that TT cells show migration *in vitro*.

4.10 Inhibition of invasion

The invading behavior of TT cells was studied through Matrigel™ invasion assays. Migration over a PET membrane covered with Matrigel, a basement membrane resembling matrix, was significantly ($p<0.05$) inhibited when 0.1µM shikonin ($IC_{50}/10$) were added to the cell culture medium and very significantly ($p<0.01$) when 0.2µM shikonin ($IC_{50}/5$) were added (Fig. 34).

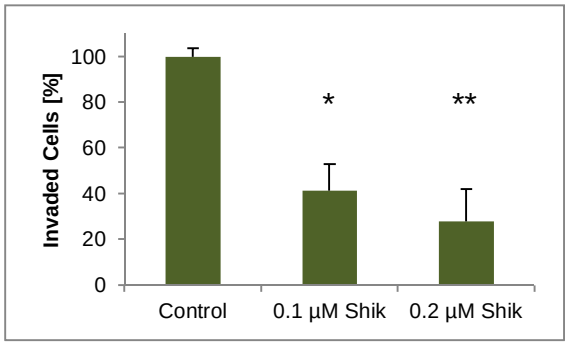


Fig. 34 – CELL INVASION IS INHIBITED BY SHIKONIN. Treatment with the $IC_{50}/10$ concentration of shikonin resulted in a significant (*) reduction of invaded cells whereas treatment with $IC_{50}/5$ led to a very significant (**) reduction compared to untreated control cells. Statistical analysis was performed using the two-tailed unpaired student's t-test.

4.11 Expression levels of MMP9 and MTDH

Quantitative real-time PCR was performed on MTC cell lines after treatment with various shikonin derivatives. Expression levels of MMP9 were measured after 4 and 24 hours of treatment (Fig. 35).

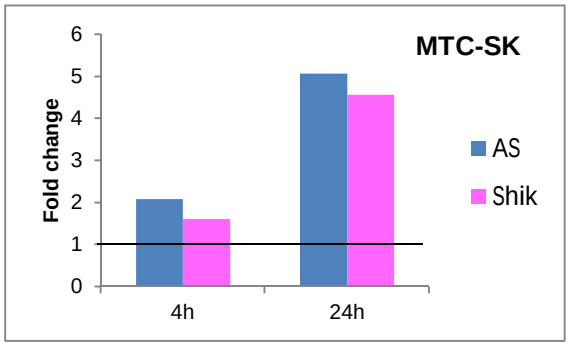


Fig. 35 – MMP9 EXPRESSION IS UP-REGULATED BY SHIKONIN DERIVATIVES. In MTC-SK cells, expression levels of MMP9 mRNA are up-regulated after treatment with 0.3µM (IC_{50}) acetylshikonin or 0.3µM (IC_{50}) shikonin compared to untreated control cells.

MMP9 expression levels were found to be up-regulated in MTC-SK cells after treatment with the respective IC_{50} concentrations of acetylshikonin and shikonin. Cells were incubated with the substances for 4 and 24 hours, respectively, before the RNA was isolated. A longer incubation period appears to contribute to the up-regulation of MMP9 mRNA expression.

Previous work carried out by Prof. Ghaffari's lab suggested that SHER-I cells do not express MMP9 and those findings were confirmed by qPCR. TT cells, likewise, did not show any mRNA expression of MMP9.

Another gene known for its crucial role in metastasis is metadherin (MTDH). Down-regulation of MTDH is associated with a reduction of metastatic seeding and a lower resistance to chemotherapy. Unfortunately, treatment with shikonin derivatives did not have any impact on MTDH expression levels in MTC cell lines (Fig. 36).

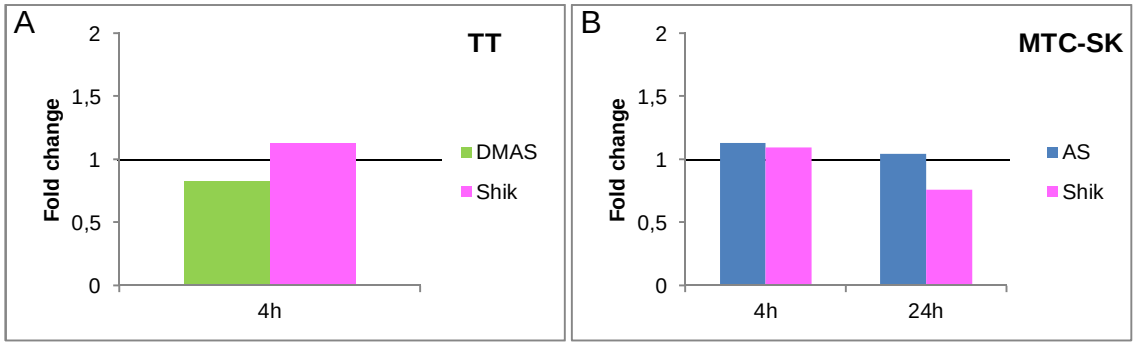


Fig. 36 – MTDH EXPRESSION LEVELS. Treatment with shikonin derivatives for 4 or 24 hours did not reduce the expression of MTDH in MTC-SK and TT cells significantly when compared to untreated control cells.

5. DISCUSSION

Neuroendocrine tumors of the thyroid account for 5-10% of all thyroid malignancies and 14% of thyroid-cancer related deaths. Currently, the only potentially curative treatment is the early surgical removal of all neoplastic tissue in the neck by total thyroidectomy. However, at least one quarter of the patients have distant metastases at the time of initial diagnosis and disease is recurring. MTC generally shows low response rates to radio- and chemo-therapy [2], [6], [9], [10], [60], [61].

Shikonin derivatives, natural products, have been used in traditional Chinese medicine for thousands of years [19], [20]. Currently, it has been reported that they are able to induce apoptosis in various cancer cell lines [26], [28], [29], [33]–[38], [47], [48].

In the present study, for the first time the effect of various shikonin derivatives on medullary thyroid carcinoma cell lines was examined. A time- and dose-dependent inhibition of proliferation by the pure derivatives shikonin, acetylshikonin and dimethylacrylshikonin, and a petroleum ether extract of *Onosma paniculata* was observed with suspension cell lines MTC-SK and SHER-I. Adherently growing cell line TT also showed a dose-dependent inhibitory effect on cell proliferation with all tested compounds. IC_{50} values were found to be ≤ 0.5 µM for suspension cell lines with shikonin and acetylshikonin being the most potent substances. SHER-I cells were more susceptible to all applied shikonin derivatives than MTC-SK. A possible explanation hereof could be that SHER-I cells originate from a primary tumor, whereas MTC-SK cells are derived from a lymph node metastasis. For adherent cells IC_{50} values ranged from approximately 1 – 2 µM with shikonin and dimethylacrylshikonin showing the biggest effect. In general, IC_{50} values were found to be lower after 72 hours than after 48 hours of incubation with the compounds indicating that the exerted effect persisted over the duration of the experiment.

These observations were also confirmed by cell viability measurements which revealed a time- and dose-dependent reduction of mitochondrial activity in all MTC cell lines. Normal human skin fibroblasts, HF-SAR, on the other hand did not show any reduction of cell viability when concentrations corresponding

to the IC_{50} values determined for MTC-SK were applied. One aspect in the search for new treatments against cancer is to find substances that specifically trigger anticancer effects and induce apoptosis in tumor cells, but not in normal cells. The findings that shikonin derivatives did not reduce cell viability significantly in normal human skin fibroblasts are thus very promising.

Suspension cell lines derived from MTCs tend to form multicellular aggregates *in vitro*. It is hypothesized that there might be a correlation between the formation of these spheroids and the resistance of NET to radio- and chemotherapy [62] and that dissociation of the cell clusters would make them more accessible for cytotoxic agents. Here, it is shown that shikonin derivatives did indeed have an effect on the morphology of cells grown *in vitro*. Application of the IC_{50} concentrations of the compounds inhibited the formation of multicellular aggregates in MTC-SK and strongly reduced it in SHER-I cells. TT cells in turn lost their polygonal shape and exhibited a rather round shape while detaching from the culture vessel.

As mentioned above, the desired outcome in potential anti-cancer drug research is the induction of apoptosis in tumor cells. Apoptosis is a highly regulated, immensely complex process of programmed destruction. Initiated by either extracellular ligands binding to cell surface death receptors or internal signals like DNA damage, apoptotic pathways lead to the activation of caspases, who are considered the central executioners of apoptosis. Morphological changes in cells sentenced to undergo apoptosis are cytoplasmic condensation, chromatin condensation, membrane blebbing and the formation of apoptotic bodies. Biochemical mechanisms in apoptotic cells include the activation of nucleases, flipping of phosphatidylserine from the cytosolic to the extracellular part of the plasma membrane and cell cycle arrest [43]–[46].

The induction of apoptosis in MTC cell lines by shikonin derivatives was confirmed by different experimental setups. Firstly, the formation of apoptotic bodies was detected by Hoechst staining of cell nuclei. Fluorescence microscopy revealed cell shrinkage, chromatin condensation and nucleic fragmentation. Secondly, caspases 3/7 activity was found to be increased 6 hours after the application of the IC_{50} concentration of the respective shikonin derivative. The activity of these effector caspases was even higher after 12 hours of incubation but decreased again after 24 hours.

Activation of the apoptotic pathway could further be demonstrated by flow cytometry. Phosphatidylserine residues are normally located on the cytosolic side of the plasma membrane but flip to the cell surface upon induction of apoptosis. In healthy tissue this is a sign for macrophages to digest the cell. In research, however, Annexin V binding to PS is used to detect apoptotic changes in cells.

Results show that acetylshikonin and shikonin induced apoptosis in MTC-SK and SHER-I cells in a time- and dose-dependent way. In MTC-SK cells, 6 hours after the application of shikonin derivatives only a slight effect was detected, indicating that apoptosis had only been induced in a small proportion of cells yet. After 12 hours of treatment, though, the percentage of apoptotic cells was increased remarkably. This effect was even enhanced after 24 hours and especially, if the $2xIC_{50}$ concentrations were applied. Interestingly, when comparing acetylshikonin and shikonin, it seems like AS has a stronger effect on the induction of apoptosis than shikonin when cells are incubated with the IC_{50} concentrations whereas shikonin seems to have a stronger effect then AS at $2xIC_{50}$ concentrations. It is also noteworthy that the proportion of apoptotic cells decreased again after 48 hours, indicating a recovery of the surviving cells. MTC-SK cells have recently been shown to possess the ability to recover damaged mitochondria through autophagy (unpublished data, courtesy R. Fuchs), especially at lower passage numbers. It can thus be hypothesized that the applied concentrations of shikonin derivatives allow one part of the cells restore their mitochondrial activity after autophagy of impaired mitochondria whereas the other part is sentenced to death.

SHER-I cells also showed a relatively low effect after 6 hours of treatment, but again, fluorescence levels of apoptotic cells were drastically increased after 12 hours. In contrast to MTC-SK, incubation of SHER-I for 24 hours did not result in higher rates of apoptotic cells indicating that the induction of apoptosis was concluded within 12 to 24 hours after application of the shikonin derivatives.

In general, flow cytometry results are in good accordance with caspase activity measurements. Both suggest the induction of apoptosis in MTC cell lines within 6 to 12 hours of treatment with shikonin compounds.

Cell cycle analysis of MTC cell lines provided further evidence for the activation of apoptotic pathways by shikonin derivatives. Application of the corresponding IC_{50} values of acetylshikonin and shikonin resulted in a cell cycle arrest of both MTC-SK and SHER-I cells. The percentage of cells in G2/M as well as in the S-phase decreased, whereas the percentage of cells in G0/G1 increased. This effect was even enhanced after the application of the $2xIC_{50}$ concentrations and led to the formation of a sub-G0 peak after 24 hours of incubation with the compounds.

Another event occurring during apoptosis is the breakdown of the mitochondrial transmembrane potential ($\Delta\Psi m$). A commonly accepted explanation hereof is that the activation of apoptotic pathways leads to the activation of Bcl-2 family members which then shuttle from the cytosol to the outer mitochondrial membrane. There they undergo conformational changes that result in the release of pro-apoptotic factors from the mitochondrial intermembrane space which in turn leads to the breakdown of $\Delta\Psi m$ [63], [64].

A different mechanism for the breakdown of $\Delta\Psi m$, especially after shikonin treatment, was postulated by Wiench, B. et al [38]. Basing their argumentation on a publication about the depolarization of the mitochondrial inner membrane after redox cycling of naphthoquinones [40], they claim that shikonin, a naphthoquinone derivative, enters the redox cycle in mitochondria. Thereby, the quinone is metabolized to a semiquinone through a one-electron reduction and then re-oxidized by molecular oxygen, resulting in the associated production of ROS. Excessive ROS accumulation eventually leads to the breakdown of the mitochondrial transmembrane potential, and the activation of apoptotic pathways.

In the present study, a breakdown of $\Delta\Psi m$ in MTC-SK cells after application of acetylshikonin was observed by means of the decrease of red JC-1 fluorescence. However, the green JC-1 fluorescence also decreased with increasing concentrations of AS. For SHER-I cells, the measurement of $\Delta\Psi m$ was not even feasible since artifacts derived from "naturally dead cells" present even in the control sample influenced the experiment.

Literature research and discussions with other researchers concluded that JC-1 staining is a suboptimal method for the examination of mitochondrial transmembrane potential breakdown after application of shikonin derivatives.

First of all, the presence of a large amount of dead cells (up to ~35%) even in the control sample interferes with the measured fluorescence intensities. Secondly, the emission spectra of JC-1 and shikonin overlap at the detected wave length and finally, quenching might also play a role in causing these artifacts [B. Wiench, personal communication].

Recently, it has been reported that shikonin inhibits tumor invasion of human high-metastatic adenoid cystic carcinoma cells via down-regulation of matrix metalloproteinase 9 (MMP-9) [30], which is known to play a critical role in tumor cell invasiveness. We found that migration and invasion of TT cells were indeed inhibited by sub-toxic concentrations (IC_{50}/5 and/or IC_{50}/10) of the various shikonin derivatives. However, quantitative real-time PCR performed on TT cells revealed that they do not show any mRNA expression of MMP9. Since down-regulation of MMP9 can thus impossibly be the reason for the inhibition of migration and invasion of TT cells, it is hypothesized that accumulated intracellular ROS lead to the release of Ca^{2+} from calcium stores like mitochondria, as proposed by Wiench, B. et al [38]. Increased $[Ca^{2+}]_i$ destabilizes the ends of growing microtubules thereby inhibiting cytoskeleton dynamics and thus cell migration [65].

Another completely different explanation is proposed by Wang, H. et al [66]. They found that invasion and metastasis of non-small cell lung cancer A549 cells is inhibited by shikonin through down-regulation of integrin β1 expression.

Since the formation of multicellular aggregates of suspension cell lines MTC-SK and SHER-I was found to be inhibited after application of shikonin derivatives, expression levels of MMP9 and metadherin (MTDH) were examined. Down-regulation of metadherin is associated with the reduction of metastatic seeding and a lower resistance to chemotherapy [67]. Application of the IC_{50} concentrations of shikonin derivatives, however, did not have any influence on MTDH expression levels in TT or MTC-SK cells.

Surprisingly, treatment of MTC-SK cells with the corresponding IC_{50} values of acetylshikonin and shikonin resulted in an up-regulation of MMP9 expression. Hence, it can be concluded that the down-regulation of MMP9 is cell line specific and not a general mode of action executed by shikonin as

suggested by Min, R. et al [30]. This statement is also supported by the findings that MMP9 is not down-regulated by shikonin in U937 lymphoma cells either [38].

SHER-I cells, like TT, were found to not express MMP9 mRNA at all. A possible explanation therefor could be that MMP9, which plays a role in many cellular processes involved in tumorigenesis – for example cell migration and metastasis – is only expressed in cell lines derived from metastases, like MTC-SK, but not in cell lines originating from primary tumors, i.e. SHER-I and TT. Another theoretical reason could be that SHER-I and TT express alternatively spliced versions of MMP9 mRNA that cannot be amplified with the primers used.

Repeated exposure of cancer cells to chemotherapeutic agents would eventually lead to the development of cancer drug resistance. Shikonin, however, has been reported to barely induce resistance in cell lines treated repeatedly over a course of 18 months. Additionally, it prevented cells from acquiring resistances to cisplatin and paclitaxel, when co-treated. Shikonin also appeared to be highly effective against known multidrug-resistant cell lines [68].

The targeted application of *Onosma paniculata* extracts and their active constituents could thus offer a new option for treatment of chemo- and radio-resistant medullary thyroid carcinoma.

6. CONCLUSION

The initial step in attempting to find new treatment options for a disease is to establish suitable models. By setting up a primary culture of a tumor derived from a patient diagnosed with FMTC and cultivating it up to date, with the intention of eventually obtaining a continuous MTC cell line, a contribution to that matter was made.

Furthermore, the present study demonstrates the antitumor effects of a petroleum ether extract of the dried roots of *Onosma paniculata* and the pure shikonin derivatives acetylshikonin, dimethylacrylshikonin and shikonin on medullary thyroid carcinoma cell lines. The induction of apoptosis at relatively low concentrations was demonstrated through different methods such as fluorescence microscopy, caspase activity measurements and various means of flow cytometry. Migration and invasion of MTC cells derived from a patient with MEN2A – TT cells – were significantly inhibited. Normal human skin fibroblasts on the other hand were not impaired at the applied concentrations of shikonin derivatives, indicating a lack of side effects.

Shikonin has already been proven effective in numerous animal studies [19], [29] and in one clinical trial, where a shikonin-containing mixture was considered safe and efficient in the treatment of later stage lung cancer [69].

Taking together the results of this *in vitro* study and the findings of the above mentioned trials, targeted application of shikonin derivatives suggests a potential clinical effect in the treatment of medullary thyroid carcinoma.

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APPENDIX: CONGRESS CONTRIBUTIONS

Abstract for ECPS 2013

The following abstract was accepted for an **oral presentation** at the 30th Meeting of the European Cell Proliferation Society (ECPS) held from April, 11th-14th 2013 in Innsbruck / Austria.

Anti-proliferative effects of shikonin derivatives on medullary thyroid carcinoma cell lines

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Objectives: Medullary thyroid carcinoma (MTC) is a calcitonin-producing neuroendocrine tumor arising from parafollicular C-cells of the thyroid gland. Since these tumors are known for their poor response to standard chemotherapy and radiotherapy, surgical intervention remains the only curative treatment. Therefore, there is a substantial need to establish new therapeutic options in the clinical treatment of these tumors.
Shikonin and its derivatives are used in traditional Chinese medicine against various diseases. Recently it has been shown that they also exhibit anti-cancer effects in several tumor cell lines. The aim of this study is to investigate the effects of shikonin derivatives isolated from *Onosma paniculata* on MTCs.

Materials and methods: MTC cell lines MTC-SK, SHER-I and TT were incubated for 24, 48 and 72 hours with different concentrations of shikonin, its derivatives acetylshikonin and dimethylacrylshikonin and a petroleum ether extract of the roots of *O. paniculata* containing several derivatives. After incubation, cells were analysed using cell counting and WST-1 cytotoxicity assay.

Results: This study shows anti-proliferative effects of several shikonin derivatives on MTC cell lines MTC-SK, SHER-I and TT. An inhibition of cell proliferation, as well as a decrease in cell viability was observed.

Conclusion: The targeted application of *O. paniculata* extracts and active constituents can be a new option in the treatment of chemoresistant and radioresistant neuroendocrine tumors.

Abstract for ISC 2013

The following abstract was accepted as a poster at the 1st International Student Congress held from July, 4th-6th 2013 in Graz / Austria. The poster was awarded with the prize **"First Place of all Poster Presentations"**.

Anti-proliferative effects of shikonin derivatives on medullary thyroid carcinoma cell lines

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Objectives: Medullary thyroid carcinoma (MTC) is a calcitonin-producing neuroendocrine tumor arising from parafollicular C-cells of the thyroid gland. Since these tumors are known for their poor response to standard chemotherapy and radiotherapy, surgical intervention remains the only curative treatment. Therefore, there is a substantial need to establish new therapeutic options in the clinical treatment of these tumors.
Shikonin and its derivatives are used in traditional Chinese medicine against various diseases. Recently it has been shown that they also exhibit anti-cancer effects in several tumor cell lines (Rinner et al., 2010).

Aim: The aim of this study is to investigate the effects of shikonin derivatives isolated from *Onosma paniculata* on MTCs.

Materials and methods: MTC cell lines MTC-SK, SHER-I and TT were incubated for 24, 48 and 72 hours with different concentrations of shikonin, its derivatives acetylshikonin and dimethylacrylshikonin and a petroleum ether extract of the roots of *O. paniculata* containing several derivatives. After incubation, cells were analysed using cell counting and WST-1 cytotoxicity assay.

Results: This study shows anti-proliferative effects of several shikonin derivatives on MTC cell lines MTC-SK, SHER-I and TT. An inhibition of cell proliferation, as well as a decrease in cell viability was observed.

Conclusion: The targeted application of *O. paniculata* extracts and active constituents can be a new option in the treatment of chemoresistant and radioresistant neuroendocrine tumors.

Influence on medicine: By discovering drugs that exhibit an anti-proliferative effect on MTC cell lines, a potential new treatment of chemo- and radioresistant neuroendocrine tumors is depicted.

Abstract for OEGMBT 2013

The following abstract was accepted for the 5th OEGMBT Annual Meeting 2013 held from September, 25th-27th 2013 in Innsbruck / Austria.

Shikonin derivatives induce apoptosis in medullary thyroid carcinoma cell lines

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Medullary thyroid carcinoma (MTC) is a calcitonin-producing neuroendocrine tumor arising from the parafollicular cells of the thyroid gland. Since these tumors are known for their poor response to standard chemotherapy and radiotherapy, surgical intervention remains the only curative treatment. Therefore, there is a substantial need to establish new therapeutic options in the clinical treatment of these tumors. Shikonin and its derivatives are used in traditional Chinese medicine against various diseases. Recently it has been shown that they also exhibit anti-cancer effects in several tumor cell lines[1] and induce apoptosis[2]. The aim of this study is to investigate the effects of shikonin derivatives isolated from *Onosma paniculata* on MTC cell lines (MTC-SK, SHER-I, TT). Thus, cultured cells were incubated with shikonin, its derivatives acetylshikonin and dimethylacrylshikonin, and a petroleum ether extract of the roots of *O. paniculata* containing several derivatives for varying amounts of time. After incubation, IC_{50} values of the compounds were determined by CASY cell counting and viability was assessed by WST-1 tests. Morphological changes and the induction of apoptosis in a caspase dependent manner were detected at relatively low concentrations (<1µM). On the contrary, when applied to normal human skin fibroblasts, these concentrations show a much smaller impact. In conclusion, the targeted application of *O. paniculata* extracts and active constituents can be a new option in the treatment of chemo- and radioresistant neuroendocrine tumors.

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