Delineating cellular and molecular mechanisms of toxicity of an extract of khat (*Catha edulis* Forsk.) in leukemia and normal peripheral blood cells

Therese Bredholt

Dissertation for the degree philosophiae doctor (PhD)

at the University of Bergen

2010

Scientific environment

This work was performed at the Gade Institute, Section for Pathology, and the Institute of Medicine, Hematology Section, Faculty of Medicine and Dentistry, University of Bergen and was funded by the University of Bergen. Paper II was supported in part by a Helse-Vest grant and Paper III in part by the Norwegian Research Council program in functional genomics.

Acknowledgements

I wish to express my gratitude to my supervisors Anne Christine Johannessen, Olav Karsten Vintermyr and Bjørn Tore Gjertsen for giving me the opportunity to take this PhD. It has truly been some interesting and educational years, which have prepared me for future tasks and challenges, and inspired me to continue within research. I would especially like to thank Bjørn Tore Gjertsen for his never-ending enthusiasm, encouragement and his ability to see what others might not see… at the time.

I would like to thank all my colleagues in the Gjertsen lab, including its previous and present members. You are all very much present and create a productive and lively environment! I would especially like to thank Siv Lise Bedringaas, Lena Fjeldstad Hansen, Ingvild Haaland and Sigrun M. Hjelle for being good and hard-working colleagues! I have really appreciated the support and encouragement from Line Wergeland, who is always helpful and very efficient! Further, I would like to thank my co-authors Lars Herfindal, Elisabeth Ersvær, Hanne Hagland, Kjell O. Fossan, Jørn Skavland, Bjarte S. Erikstein and Karl J. Tronstad. I would also like to thank Stein O. Døskeland and the members of his group, and the members of the Bruserud lab. I have really appreciated my previous and present office 'mates' Malin V. Jonsson, Daniela E. Costea and Joachim H. Dahl and acknowledge them for excellent daily conversations, frequently of non-scientific nature. I am sending thanks and acknowledgements all the way to Kenya to my co-authors and previous colleagues Ochiba M. Lukandu and Elizabeth A.O. Dimba, for the good collaboration we have had on the khat project.

Friends deserve to be thanked for their support and understanding, and for still being around! Finally, I would like to express my sincere gratitude to my parents Britt and Hans Bredholt for their support and care, for listening to details regarding my work, and for taking care of lovely Leia.

Bergen, January 2010

Therese Bredholt

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Abstract

Plants have been used in treatment and prevention of diseases for thousands of years. In modern medicine there is a trend towards isolation and identification of bioactive molecules, whereas plant preparations continue to be used in traditional medicinal systems. However, combinations of drugs are usually administered in treatment of complex diseases like cancer and HIV, in order to target multiple deregulated cellular pathways simultaneously.

Khat (*Catha edulis* Forsk.) has been cultivated for centuries primarily due to its use as a natural stimulant. The stimulating potential of khat is mainly caused by its content of cathinone, an alkaloid with structure and pharmacological profile similar to amphetamine. Cathinone is a labile precursor for the less bioactive derivatives cathine and norephedrine. An organic extract of khat was previously reported to induce programmed cell death in acute myeloid leukemia (AML) cell lines, while being less toxic to normal peripheral blood mononuclear cells (PBMCs). In this study, cellular and molecular effects of an extract of khat were further elucidated in AML cell lines and compared with the cancer therapeutics camptothecin. Early effects of khat and the khat amphetamines on intracellular signaling responses in normal peripheral leukocytes were investigated, in addition to cytotoxic effects. The khat extract was fractionated and analysed for cytotoxicity in AML cell lines, and the fractions analysed by mass spectrometry.

The khat extract was shown to induce cell death in a subset of genetically diverse AML cell lines, indicating involvement of specific mechanisms. In contrast to camptothecin, khat caused structural damage to mitochondria and mediated impaired mitochondrial respiration. In addition, khat was observed to induce formation of autophagosomes, indicating activation of autophagy. The survival protein Bcl-2 protected against camptothecin, and partial protection was obtained against khat-induced cell death. Procaspase-8 of the receptor-mediated cell death pathway was activated by khat, while levels of the death antagonists Mcl-1 and c-FLIP$_L$ were reduced. The stress sensor and tumour suppressor protein p53 was induced and modulated in khat-mediated death, but not by sub-lethal dilutions of the khat extract or in khat-resistant cells. Experiments using p53 knock-down and knock-out cells demonstrated that khat-mediated cell death was independent of p53. The p53 protein was

suggested to primarily act as a stress sensor in AML cells that were susceptible to khat-induced cell death.

Fractionation of the khat extract and bio-guided screening in AML cell lines resulted in identification of three separate cytotoxic fractions. The khat fractions were analysed by mass spectrometry, which led to the partial characterization of a phenylpropanoid glycoside suggested to represent the major cytotoxic constituent in the khat extract. In contrast to khat, the khat amphetamines were observed to be relatively non-toxic to AML cell lines. Khat and the khat amphetamines were shown to mediate early and generally opposite effects on signaling mediators in normal immune cells. Whereas khat activated stress sensors, like p38 and p53, and demonstrated cytotoxic effects, the khat amphetamines attenuated activating modifications of several signaling proteins, including p53, and appeared to have a stimulating effect on lymphocyte proliferation.

Sammendrag

Planter har blitt brukt i behandling og forebygging av sykdom i tusener av år. I moderne medisin er det vanlig å isolere og identifisere bioaktive substanser, mens plantepreparater fortsatt blir brukt i tradisjonelle medisinske systemer. Kombinasjoner av ulike typer medisin er likevel vanlig ved behandling av sykdommer som kreft og HIV, med den hensikt å ramme flere av de deregulerte cellulære signalveiene som forårsaker sykdom.

Khat (*Catha edulis* Forsk.) har blitt dyrket i århundrer, først og fremst for å bli benyttet som en naturlig stimulant. Den stimulerende effekten skyldes primært khat plantens innhold av katinon, et alkaloid som har lignende struktur og farmakologisk profil som amfetamin. Katinon er en labil forbindelse som blir omdannet til de mindre stimulerende derivatene katin og norefedrin. Et organisk ekstrakt av khat ble tidligere vist å aktivere programmert celledød i akutt myelogen leukemi (AML) cellelinjer, mens det var mindre toksisk for normale perifere blod mononukleære celler (PBMC). I denne studien ble de cellulære og molekylære effektene av et khat ekstrakt videre undersøkt i AML cellelinjer, og sammenlignet med kreftmedisinen camptothecin. Tidlige effekter av khat og khat amfetaminene på intracellulære signaleringsproteiner ble undersøkt i normale leukocytter, i tillegg til cytotoksiske effekter. Khat ekstraktet ble fraksjonert og undersøkt for cytotoksiske effekter i AML cellelinjer, og fraksjonene ble analysert med massespektrometri.

Khat ekstraktet ble vist å indusere celledød i et utvalg av genetisk ulike AML cellelinjer, noe som indikerte aktivering av spesifikke mekanismer. I motsetning til camptothecin førte khat behandling til strukturell skade i mitokondriene, noe som hemmet den mitokondrielle respirasjonen. Videre ble det observert at khat induserte dannelse av autofagosomer, noe som indikerte aktivering av autofagi. Overlevelsesproteinet Bcl-2 beskyttet mot camptothecin, men ga bare delvis beskyttelse mot khat-indusert celledød. Procaspase-8, som er del av den reseptormedierte celledødsveien, ble aktivert av khat, mens nivåene av dødsantagonistene Mcl-1 og c-FLIP$_L$ ble redusert. Stress sensor og tumor suppressor proteinet p53 ble indusert og modulert i khat-mediert død, med ikke av ikke-dødelige khat doser eller i khat-resistente celler. Eksperimenter med p53 knock-out og knock-down celler viste at p53 ikke var nødvendig i khat-mediert celledød. p53 proteinet ble foreslått å fungere primært som en stress sensor i AML celler som var sensitive for khat.

Fraksjonering av khat ekstraktet og testing av fraksjonene for toksisitet i AML cellelinjer, resulterte i identifisering av tre separate cytotoksiske fraksjoner. Khat fraksjonene ble analysert med massespektrometri, og et fenylpropanoid glykosid ble delvis karakterisert og foreslått å være den mest toksiske komponenten i khat ekstraktet. In motsetning til khat ble khat amfetaminene vist å være relativt ikke-toksiske i khat-sensitive AML celler. Khat og khat amfetaminene ble vist å gi hurtige og generelt motsatte effekter på ulike signaleringsproteiner i normale immun celler. Mens khat aktiverte stress proteiner, som p38 og p53, og induserte cytotoksiske effekter, resulterte khat amfetaminene i reduserte nivåer av aktiverende modifikasjoner hos flere signalproteiner, inkludert p53, og ble vist å ha en stimulerende effekt på lymfocytt proliferasjon.

List of papers

Paper I

Therese Bredholt, Elizabeth A.O. Dimba, Hanne R. Hagland, Line Wergeland, Jørn Skavland, Kjell O. Fossan, Karl J. Tronstad, Anne C. Johannessen, Olav K. Vintermyr and Bjørn T. Gjertsen. **Camptothecin and khat (Catha edulis Forsk.) induced distinct cell death phenotypes involving modulation of c-FLIP$_L$, Mcl-1, procaspase-8 and mitochondrial function in acute myeloid leukemia cell lines.**

Mol Cancer 2009, 8(1):101

Paper II

Therese Bredholt, Sigrun M. Hjelle, Ingvild Haaland, Line Wergeland, Sjur Huseby, Olav K. Vintermyr, Anne C. Johannessen and Bjørn T. Gjertsen. **Modulation of p53 isoforms and its post-translational modifications reflect the cytotoxicity of a botanical khat extract.**

Manuscript

Paper III

*Therese Bredholt, *Elisabeth Ersvær, Bjarte S. Erikstein, Kjell O. Fossan, Anne C. Johannessen, Olav K. Vintermyr, Øystein Bruserud and Bjørn T. Gjertsen. **Natural khat-derived amphetamines attenuate phosphorylation of AKT, STAT6, CREB and p53 in peripheral leukocytes.**

Manuscript

Paper IV

Therese Bredholt, Lars Herfindal, Andrew Marston, Anne C. Johannessen, Bjørn T. Gjertsen and Olav K. Vintermyr. **Bio-guided isolation of a major cytotoxic constituent in khat (*Catha edulis* Forsk.) unrelated to natural khat amphetamines.**

Manuscript

*Therese Bredholt and Elisabeth Ersvær are equal first authors.

Abbreviations

AIF	apoptosis inducing factor
AML	acute myeloid leukemia
ATP	adenosine triphosphate
Bax	Bcl-2 associated X protein
Bcl-xL	B-cell lymphoma extra long
Bcl-2	B-cell lymphoma gene 2
BH	Bcl-2 homology
BID	BH3 interacting domain death agonist
Caspase	cysteine aspartate proteinase
CHX	cycloheximide
CPT	camptothecin
CREB	cAMP response element binding
DISC	death inducing signaling complex
DMSO	dimethylsulphoxide
ERK	extracellular signal-regulated kinase
FLICE	FADD-like IL-1β-converting enzyme
FLIP	FLICE inhibitory protein
FLT3	fms-like tyrosine kinase 3
GS-MS	gas chromatography-mass spectrometry
HPLC	high pressure liquid chromatography
IM	inner membrane
IMS	inter membrane space
MAPK	mitogen-activated protein kinase
Mcl-1	myeloid cell leukemia-1
Mdm2	minute double murine 2
MLL	multiple lineage leukemia
MOMP	mitochondrial outer membrane permeabilization
MPT	mitochondrial permeability transition
MS	mass spectrometry
OM	outer membrane

PBMC	peripheral blood mononuclear cell
PFA	paraformaldehyde
PMA	phorbol myristate acetate
PTM	post translational modification
PTP	permeability transition pore
PUMA	p53-upregulated modulator of apoptosis
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
STAT	signal transducer and activator of transcription
TEM	transmission electron microscopy
VDAC	voltage dependent anion channel
WST-1	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt

1. INTRODUCTION

1.1 Plants in medicine

Plants have been used for both medicinal and recreational purposes for thousands of years. Medicinal plants were traditionally used as crude preparations, like teas and powders, in treatment and prevention of diseases. In modern Western medicine the drug discovery process from plants typically involves isolation and characterization of the active component(s) from crude extracts. The concept of isolating bioactive components started with the preparation of the alkaloid morphine from opium in the early 19th century. In Western medicine it is conventional to use single ingredient drugs or combinations of several characterized drugs. The combination therapies are designed to target several pathological pathways simultaneously, thereby increasing treatment efficacy. Combination strategies have proven particularly suited for treatment of infectious diseases like HIV, tuberculosis and malaria, and in therapeutic interventions for complex chronic diseases like cancer and metabolic syndrome (1, 2).

It has been reported that 25% of all drugs prescribed today are derived from plants. Out of many families of secondary metabolites, nitrogen-containing alkaloids have contributed the largest number of drugs to the modern pharmacopeia. Most drugs isolated from plants or that are derivatives thereof are employed in the fight against cancer (1, 2). Anti-cancer drugs from plants in current use can be categorized into four main classes of compounds: vinca alkaloids, epipodophyllotoxins, taxanes and camptothecins (1). The process of drug discovery has been estimated to take an average of 10 years upwards. Drug discovery from plants has traditionally been an even slower process, being inherently more complicated than modern drug discovery techniques. As a result, pharmaceutical companies have been observed to eliminate or scale down their natural product research over the last decades (3).

Today the identification of compounds with therapeutic potentials is typically based on high-throughput screening (HTS) platforms, which in part relies on the use of cell-free bio-assays. However, there has recently been more emphasis on development of cell-based screening techniques. One of the techniques that are being developed and refined is the

method of multi-parameter single cell analysis of intracellular signaling pathways (4). The development of technical platforms that enable more efficient identification of novel compounds form natural sources is believed to re-strengthen the interest in natural products as an invaluable source for novel therapeutics.

1.2 Khat (*Catha edulis* Forsk.) - a natural stimulant

Khat (*Catha edulis* Forsk.) is an evergreen shrub of the Celastraceae family that is cultivated primarily for its use as a natural stimulant. The habit of khat chewing is practised by millions of people, particularly in Yemen, Somalia, Ethiopia, Djibouti and Kenya, representing main regions of cultivation (5, 6). The habit is spreading to other parts of the world, but is mainly confined to immigrant communities from countries where khat chewing is endemic (6, 7). In addition to its use as a social and recreational drug, processed leaves and roots of khat are used in treatment of various conditions like influenza, cough, asthma, malaria, gonorrhoea, vomiting and headache (6, 8).

Figure 1. Bundle of khat on a banana leaf. The banana leaf is wrapped around the bundle in order to keep the leaves and shoots fresh.

Khat chewing is predominantly a social habit, which is practiced in special khat chewing sessions, where different issues are discussed and resolved. Fresh leaves and shoots of khat are chewed slowly and the juice of the material swallowed while the plant residue is retained as a quid on one side of the mouth. About 100-200 g of khat is typically chewed per

person in one session, which usually lasts for 3-4 hours, but the amount may vary extensively. Khat induces a mild euphoric state giving the chewer a feeling of being more focused, energetic and communicative. Labourers, farmers and students are known to use khat during work in order to increase alertness and reduce physical fatigue (6, 7).

1.3 The natural khat amphetamines

The stimulating effects of khat are mainly due to its content of the alkaloid S-(-)-cathinone, and to a lesser extent the diastereomers (1S,2S)-(+)-cathine and (1R,2S)-(-)-norephedrine (9, 10). Cathinone is an intermediate in the biosynthesis of cathine and is found mainly in young leaves of the khat plant. Cathinone is also converted to cathine upon wilting of the khat leaves. Khat is therefore consumed when it is fresh and it is primarily the young leaves and shoots that are harvested (9). The price of khat at markets in Ethiopia, Kenya and Yemen have been reported to correlate with the content of cathinone (11).

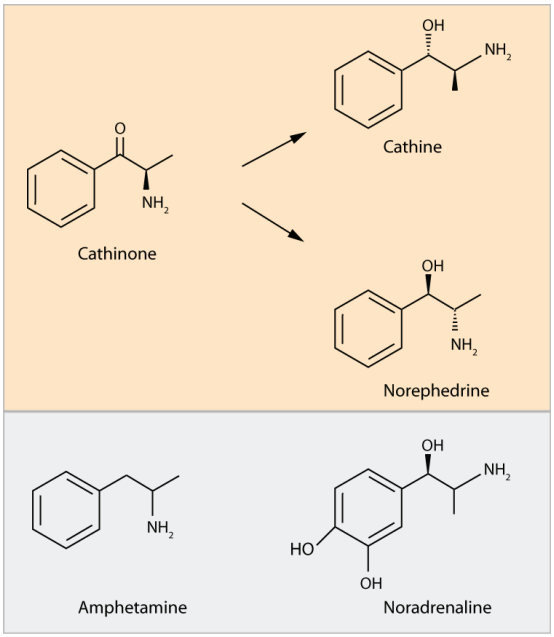

Figure 2. Structures of the natural khat amphetamines: cathinone, cathine and norephedrine, the synthetic drug amphetamine and the neurotransmitter noradrenaline.

Cathinone is structurally related to the synthetic drug amphetamine and has been shown to have a similar pharmacological profile. Due to this similarity, cathinone has been

termed a natural amphetamine and khat an amphetamine-like stimulant (5, 9). Cathinone and amphetamine induce central nervous system and peripheral effects like euphoria, hyperactivity, restlessness, mouth dryness, mydriasis, anorexia, hyperthermia, hypertension and tachycardia (5, 7, 9). The structures of cathinone and amphetamine are related to monoamine neurotransmitters, and the compounds mediate their effects mainly by acting as indirect symphatomimetics (Figure 2). Cathinone and amphetamine induce release of neurotransmitters (i.e. noradrenaline, dopamine and serotonin) from pre-synaptic neuronal terminals and inhibit their reuptake from the synaptic gap (12). Cathinone has also been shown to bind and activate α_2-adrenergic and $5HT_7$ serotonin receptors (13, 14).

The stimulating khat amphetamines are efficiently extracted into saliva during chewing, with only 10% of the compounds remaining in the plant residue (15). The oral buccal mucosa plays a major role in absorption of the khat amphetamines, but some absorption may also occur via the stomach and the small intestine. Maximal blood plasma concentrations of the amphetamines following experimental khat chewing have been measured after 2.3 hours for cathinone, 2.6 hours for cathine and 2.8 hours for norephedrine (15). The khat amphetamines can be detected in urine by gas chromatography-mass spectrometry (GS-MS), and it has been shown that less than 7% of ingested cathinone is secreted, while norephedrine is found in larger quantities than the amount ingested (16). The ratios of the khat amphetamines reflect the preferred *in vivo* metabolism of cathinone to norephedrine (17). GS-MS has also been used to determine the concentrations of the khat amphetamines in hair from khat chewers, and their concentrations were reported to reflect the level of khat consumption (18, 19).

1.4 Chemical composition of khat

The chemical composition of khat is influenced by local conditions, geographical region and the time of season in which it is grown. In addition, numerous khat variants exist which demonstrate considerable differences in general appearance and stimulating potential (7). An analysis of 22 khat samples demonstrated that 100 g fresh khat on average contains 36 mg cathinone, 120 mg cathine and 8 mg norephedrine (11). However, the concentrations of the khat amphetamines were observed to vary extensively between the different samples.

In addition to the khat amphetamines, khat contains a family of alkaloids with molecular weights in the 600-1,200 range termed cathedulins (20). Liquid

chromatography/mass spectrometry demonstrated the presence of 62 cathedulins in methanol extracts of khat (21). A third group of alkaloids has been isolated from khat grown in the Meru district in Kenya, which include the phenylpentenylamines merucathine, merucathinone and pseudomerucathine. Cathedulines and phenylpentenylamines are not believed to possess significant biological activity (22). The pigmented root-bark contains triterpenoid quinones including celastrol, pristimerin, iguesterin and tingenone (20). Tingenone has also been isolated from khat callus cultures, in addition to 22 β-hydroxytingenone (23). Khat contains polyphenols, where especially tannins have been found to be present in considerable quantities: 7-14% by weight in dried leaves depending on khat cultivate and method of estimation (5). Khat has been shown to contain β-sitosterol and its glycoside derivatives (10), in addition to flavonoids (24, 25), which include myricetin and quercetin glycosides (5). The ascorbic acid content of khat has been reported to be high, with 100 g of fresh leaves containing 130-160 mg (5). Minor constituents of khat not believed to mediate biological effects include β-carotene, thiamine, riboflavin, niacin and calcium (5).

1.5 Adverse health effects associated with habitual khat use

Khat chewing is associated with the development of oral keratotic white lesions within the vestibule or buccal mucosa, where the khat quid is placed (26). A study reported several histopathological changes, but none of the lesions were considered to be premalignant (27). However, several studies have indicated a correlation between the khat habit and development of oral cancer and squamous cell carcinomas of head and neck (28, 29). It has been suggested that khat use, in combination with alcohol and tobacco consumption, represents a potential cause of malignant transformation (30).

Khat chewers experience different gastrointestinal problems like gastritis and constipation, believed to be caused by the astringent nature of khat tannins (5). Khat chewing has been indicated as a risk factor for development of duodenal ulcers (31). The development of haemorrhoids is shown to be significantly associated with the khat habit (32). Khat mediates hypertension and tachycardia, and it has been demonstrated that chewers have an increased risk for acute myocardial infarction (AMI) (33). Cathinone has been shown to cause coronary and aortic vasoconstriction, indicating its involvement in myocardial infarction (34). Amphetamine has also been suggested to increase the incidence of AMI, and was reported after intravenous injection of the drug (35).

A range of adverse health effects associated with khat chewing has been reported, affecting the cardiovascular, gastrointestinal, hepatobiliary, genitourinary, respiratory and central nervous systems. Khat use has also been shown to mediate metabolic, endocrine, ocular and psychiatric effects. The many adverse effects associated with khat use have recently been reviewed elsewhere (6, 8, 36, 37).

1.6 Potential beneficial effects by khat and its constituents

Although khat-chewing is primarily associated with adverse health effects, positive effects of khat and its constituents have been reported. Processed leaves and roots of khat have been used to treat influenza, cough, asthma and other chest problems in areas of cultivation (6, 8). Cathinone was recently shown to inhibit acetylcholine release and contractions of smooth muscle, which could explain the use of khat as a remedy for respiratory diseases (13). Long term exposures of rabbits to khat resulted in lowered cholesterol, glucose and triglyceride concentrations in blood plasma (38). Cathine and norephedrine have been shown to accelerate sperm capacitation and inhibit spontaneous acrosome loss, indicating a beneficial effect on fertility (39). Khat users in Somalia were reported to have increased lymphocyte counts and percentage CD4 positive cells, suggesting a stimulating effect on the immune system (40). Cathinone was shown to mediate IL-2 production, B-lymphocyte proliferation and cytotoxic T-lymphocyte induction (41).

A beneficial impact on oral health has been indicated, as the caries prevalence amongst khat chewers is reported to be low. Recently, khat extracts were shown to inhibit biofilm formation by the principal cariogenic bacterium in humans, Streptococcus mutans, suggesting anti-cariogenic properties (42). In addition, khat extracts were found to possess selective anti-microbial properties, being particularly toxic to periodontal disease-associated bacteria (43). Khat flavonoids have been shown to possess significant anti-inflammatory activity in rats, comparable to the standard anti-inflammatory drug oxyphenbutazone (24). Another study demonstrated that khat flavonoids possessed antioxidant properties for oxidative stress generated during restraint stress in rats (25).

Khat contains vitamin C, which in addition to being an antioxidant has been shown to have anti-cancer effects *in vivo* in a process involving generation of reactive oxygen species (ROS) (44, 45). Compounds mediating cell death possess a toxic potential, but could also represent promising anti-cancer therapeutics. Callus cultures of khat were reported to contain

the triterpenoids tingenone and 22 β-hydroxytingenone, which demonstrated anti-bacterial activities and mediated cell death in various cancer cell lines (23). Khat also contains the triterpenoid celastrol, which has been shown to possess both anti-inflammatory and anti-leukemic activities (46). Celastrol was recently reported to induce apoptosis in leukemic stem cells, which are implicated in disease relapse (47, 48). The triterpenoid pristimerin has been shown to induce cell death by mitochondrial targeting in breast cancer cells (49), via proteosomal targeting in prostate cancer cells (50), and it has also been shown to inhibit proliferation of leukemic cells (51). An overview of khat constituents and reported beneficial effects is provided in Table 1.

Table 1. Compounds found in khat with potential therapeutic effects.

Khat constituent:	Biological effect:	Reference:
Alkaloids		
• cathinone	IL-2 production, B-lymphocyte proliferation, cytotoxic T-lymphocyte induction, acetylcholine release	(13, 41)
• cathine	acceleration of sperm capacitation, inhibition of acrosome loss	(39)
• norephedrine	acceleration of sperm capacitation, inhibition of acrosome loss	(39)
Flavonoids		
• myricetin	antioxidant, anti-cancer, anti-inflammatory	(52, 53)
• quercetin	antioxidant, anti-inflammatory	(54)
Phytosterols		
• β-sitosterol	lowers blood cholesterol, anti-cancer	(55, 56)
Sugar acids		
• ascorbic acid	antioxidant, anti-cancer	(44, 45)
Triterpenoids		
• celastrol	anti-cancer, anti-inflammatory	(46, 47)
• pristimerin	anti-cancer	(49-51)
• tingenone	anti-microbial, anti-cancer	(23)
• 22 β-hydroxytingenone	anti-microbial, anti-cancer	(23)

1.7 Toxic effects by khat and underlying mechanisms

Most studies have focused on the pharmacology of khat and the khat amphetamines, whereas relatively few reports have investigated potential cytotoxic effects and underlying mechanisms. Khat and the alkaloid fraction were shown to produce oxidative stress and toxicity in rats by reducing the levels of free radical scavenging enzymes and glucose (25). It

was recently reported that khat inhibited ROS scavenging enzymes in blood serum from human chewers, resulting in significant elevations in free radical loads (57).

Khat has been shown to affect blood formation and cause bone marrow suppression in mice, while reducing the mitotic index of somatic cells and inducing chromosomal aberrations (58). A study using the micronucleus test to evaluate khat-mediated genetic damage in humans reported that khat use, especially in combination with alcohol and tobacco, could be a potential cause of oral malignancy (30). An organic khat extract was shown to induce tumour suppressor proteins and G1 cell cycle arrest in normal oral fibroblasts and keratinocytes *in vitro* (59). When exposed to a higher concentration of khat the oral fibroblasts and keratinocytes underwent programmed cell death in a process involving ROS (60). Khat-mediated cell death in normal oral fibroblasts and keratinocytes was recently reported to involve an early effect on mitochondrial integrity and function (61).

We previously reported that an organic extract of khat induced cell death in acute myeloid leukemia (AML) cell lines *in vitro* (62). Normal peripheral blood mononuclear cells (PBMCs) were shown to be less sensitive to khat toxicity when compared to AML cell lines. The process involved caspase-activation and could be blocked by cycloheximide (CHX), an inhibitor of protein synthesis. Khat-meditated cell death was reduced by treatment with inhibitors of caspase-8, indicating involvement of cell surface death receptors. The khat amphetamines were suggested to be partly responsible for the toxic effects of khat in the HL-60 cell line (62, 63). The studies on AML cell lines and PBMCs suggested that khat could contain compounds with potential as anti-cancer therapeutics, and provided the basis for the work in this thesis. Table 2 provides an overview of toxic effects of khat extracts and suggested underlying mechanisms and/or proposed khat constituents.

Table 2. Toxic effects by khat extracts in various systems and responsible mechanisms or constituents.

Toxic effect by khat:	Biological system:	Mechanism/constituent:	Reference:
induction of cell cycle arrest	primary human oral keratinocytes and fibroblasts	activated p53, p16 and p21	(59)
induction of cell death	primary human oral keratinocytes and fibroblasts	loss of mitochondrial inner transmembrane potential, ROS	(60, 61)
induction of cell death	human acute myeloid leukemia cell lines	caspase-activation	(62)
induction of cell death	human cancer cell lines	due to tingenone and 22 β-hydroxytingenone	(23)
increased ROS	human serum, *in vivo*	inhibition of ROS scavengers	(57)
increased ROS	rat blood, *in vivo*	reduced levels of ROS scavengers, due to the alkaloid fraction?	(25)
genetic damage	human buccal and bladder mucosa cells		(30)
genetic damage	mice, *in vivo*		(58)
anti-bacterial	bacteria	due to tingenone and 22 β-hydroxytingenone	(23)
anti-bacterial	oral bacteria		(43)

1.8 Acute myeloid leukemia cell lines in toxicity studies

Cell lines are frequently used in investigations of compounds with toxic and therapeutic potentials. We previously used AML cell lines when studying cellular and molecular effects of khat extract and the khat amphetamines. In addition to representing a model system for AML, the cell lines represent systems that are easy to maintain and manipulate. Different AML cell lines that are characterized by various genetic defects are commercially available. The genetic aberrations may be used as tools when mechanisms underlying cellular effects are investigated.

AML is a hematological malignancy involving uncontrolled proliferation of myeloid progenitors that harbour a differentiation block (64). An overview of normal hematopoiesis is provided in Figure 3.

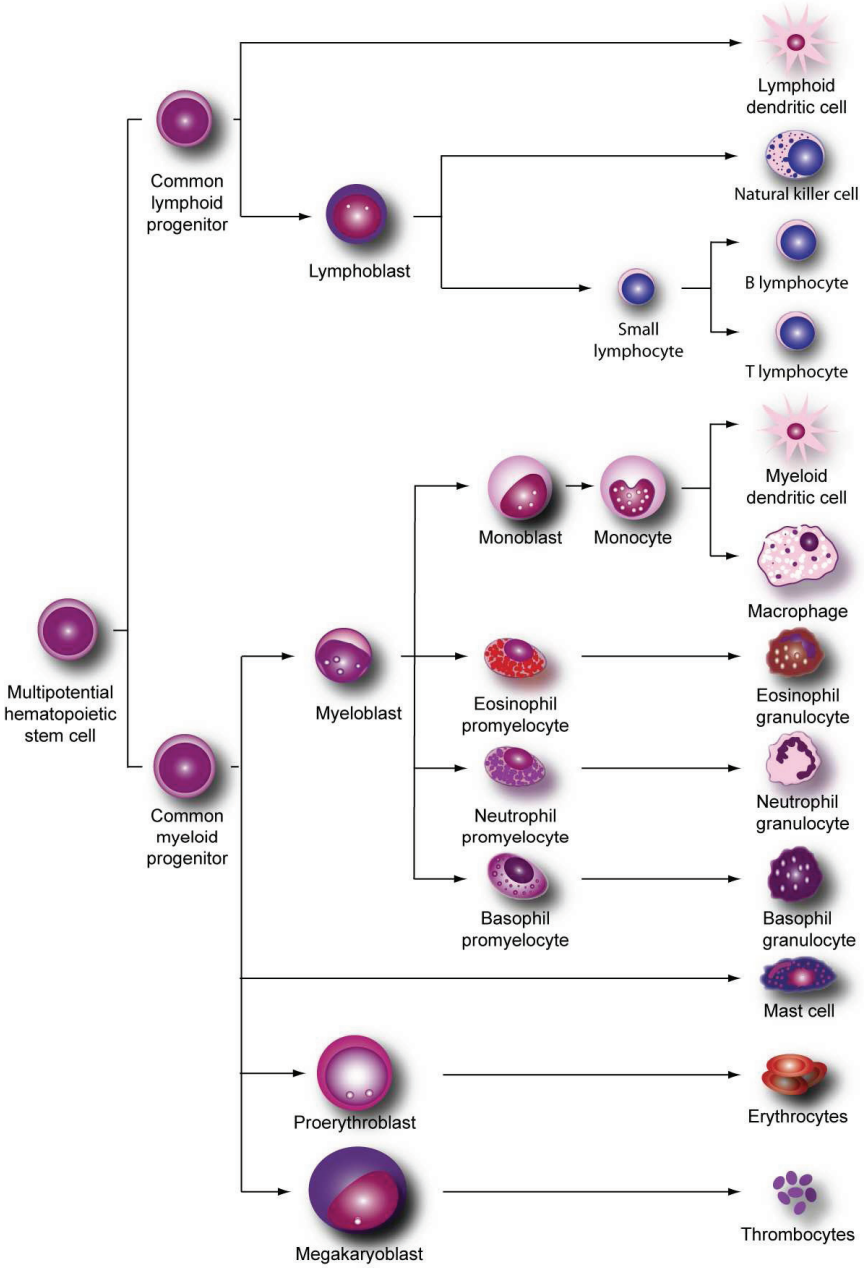

Figure 3. Overview of normal hematopoiesis: the generation of mature lineage-specific blood cells from a common hematopoietic stem cell. The figure is from Line Wergeland's PhD thesis, UoB, Bergen, Norway, and is printet with her permission.

A two-hit model for AML development has been proposed, where the genetic aberrations are divided into two complementary groups (65). Class I includes mutations that activate signal transduction pathways, resulting in enhanced proliferation/survival of leukemic progenitors. Class II comprises mutations affecting transcription factors and/or transcriptional co-regulators, resulting in impaired differentiation and escape from apoptosis. An overview of class I and class II mutations is provided in table 3.

Table 3. Overview of complementary class I and class II mutations that cooperate in development of AML. Adapted from (66).

Class I mutations	Class II mutations
BCR-ABL	CBFβ-MYH11
N-RAS	AML1-ETO
K-RAS	TEL-AML1
c-KIT (exon 8)	PML-RARα
c-KIT (Asp816)	NUP98-HOXA9
FLT3 (ITD)	PU.1
FLT3 (Asp 835)	C/CEPα
PTPN11	AML1
NF1	AML1-AMP19
TEL-PDGFRβ	MLL fusion proteins

AML is a heterogeneous disease characterized by non-random genetic defects. Approximately 55% of adult patients display chromosomal trans-localizations and cytogenetics represents the most important prognostic factor predicting therapy response and overall survival. AML patients are broadly divided into three risk groups based on cytogenetics with favourable, intermediate and adverse prognosis (67-69). Approximately 45% of AML patients present cytogenetically normal (CN) myeloblasts at the time of diagnosis. Risk stratification and determination of therapeutic strategy in CN-AML patients is based on the presence or absence of specific mutations (70). Various mutations that affect oncogenes, tumour suppressors and transcription factors are frequently found in AML. Several of these mutations represent independent prognostic factors, in addition to providing potential therapeutic targets.

1.9 Prognostic factors and therapeutic targets in AML

Mutations in the nucleophosmin 1 (*NPM1*) gene are found in 45-62% of CN-AML patients, representing the most frequent genetic alteration in this subset. The NPM1 protein is activated by phosphorylation and is involved in diverse processes related to proliferation, growth suppression and differentiation (71). Approximately 40% of patients harbouring mutations in *NPM1* also carry internal tandem duplications (ITDs) in the *FLT3* gene encoding the Fms-like tyrosine kinase 3 receptor. *NPM1* mutations represent a favourable prognostic marker, but only in the absence of *FLT3*-ITDs (70). The FLT3 receptor is constitutively activated in approximately 30% of AML patients by ITDs, representing an independent prognostic factor which confers a poor prognosis (72).

Constitutive activation of receptor tyrosine kinases (RTKs), like FLT3 and c-KIT, causes aberrant signaling via intracellular cascades meditated by phosphorylation of signaling proteins. Signaling proteins frequently found to be acitvated in cancers include members of the family of signal transducers and activators of transcription (STATs), the Ras/mitogen-activated protein kinases (MAPKs) and the PI3K/AKT pathway (73). The transcription factor STAT5 is constitutively activated in cells with *FLT3* mutations (74) and has been shown to induce transcription of survival proteins like Bcl-2 and Bcl-xL (75, 76). Over-expression of surval proteins frequently mediates chemoresistance and poor overall survival.

Several of the recurring genetic aberrations and deregulated pathways in AML represent potential therapeutic targets. Small molecules that inhibit the activity of constitutively activated RTKs are being evaluated for treatment in clinical trials, and strategies that target down-stream effects like over-expressed survival proteins are being investigated. Detailed reviews on prognostic markers and therapeutic targets in AML have recently been published (70, 73, 77).

1.10 Treatment of AML

Conventional AML treatment consists of induction therapy where a combination of an anthracycline or anthracenedione (daunorubicin, idarubicin, mitoxantrone) and cytarabine-arabinoside (cytarabine) is administered. A majority of patients receiving intensive chemotherapy will achieve complete remission, defined as less than 5% myeloblasts in the

bone marrow, after initial induction treatment (64, 78). The patients will then receive consolidation therapy with either allogeneic stem cell transplantation or repeated cycles of intensive chemotherapy. Allogeneic stem cell transplantation and high-dose cytarabine represent the most intensive therapeutic approaches, but serious side-effects and high treatment-related mortality make these interventions possible only for patients below 60 years of age (64, 78). Patients up to 70 years of age are often treated with less intensive chemotherapy and patients above 70 years usually receive supportive therapy alone, the median survival of these patients being 3-4 months (64, 78). Overall AML free survival is 40-50% even for the younger patients who receive the most intensive chemotherapy, and the most important cause of death is therapy-resistant leukemia relapse (78). AML relapse is believed to be caused by acquisition of drug resistance and/or failure of the treatment to target AML stem cells (48).

1.11 Programmed cell death

Anti-cancer interventions may be opposed by numerous genetic alterations, which frequently affect pathways of programmed cell death (79-81). The aim in therapeutic interventions is often restoration of the ability of cancer cells to undergo programmed cell death (82).

Programmed cell death was previously considered synonymous with the term apoptosis (83). Apoptosis was pictured to be induced either via an extrinsic (receptor-mediated) or an intrinsic (mitochondria-mediated) pathway, which converged on activation of cellular proteinases termed caspases (cysteine-aspartate proteinases). The caspases catalyze cleavage of cellular proteins, mediate DNA-degradation and cause cell destruction (84). However, it is now evident that programmed cell death may occur through other regulated sequences of events, with or without the involvement of caspases.

According to the recommendations by The Nomenclature Committee on Cell death 2009, programmed cell death can be broadly divided into apoptosis, autophagy and necrosis (85). The morphological features characteristic for apoptosis include reductions in microvilli, cellular and nuclear shrinkage, chromatin condensation, nuclear fragmentation, little or no ultra structural changes in cytoplasmic organelles and plasma membrane blebbing. Even if caspase-activation is no longer considered a hallmark of apoptosis, it may be necessary for acquisition of the characteristic apoptotic morphology (86).

Autophagy is characterized by the formation of authophagic vacuoles, which are double-membrane vesicles containing cellular organelles, proteins and cytoplasm. Upon fusion with lysosomes the autophagosome inner membrane and the sequestered material is degraded, resulting in recycling of building blocks and generation of cellular energy. The catabolic step marks the completion of the autophagic pathway. Autophagy is triggered in times of nutrient deprivation and also occurs as a means of degrading damaged organelles (87, 88). It primarily represents a cell survival mechanism, but has in addition been demonstrated to mediate cell death (89).

Necrosis was originally considered to be an accidental and uncontrolled mode of cell death, but has now been shown to involve regulated sequences of events (90). Morphological characteristics include gain in cell volume, swelling of organelles, plasma membrane rupture and loss of intracellular contents. An overview of morphological characteristics of the three main modes of programmed cell death is provided in Table 4.

Table 4. Simplified overview of morphological characteristic of the main modes of cell death according to the recommendations by The Nomenclature Committee on Cell death 2009 (85).

Mode of cell death:	**Morphological characteristics:**
Apoptosis	Reduction of microvilli
	Reduction of cellular and nuclear volume
	Nuclear fragmentation
	Minor modifications of cytoplasmic organelles
	Plasma membrane blebbing
Autophagy	Lack of chromatin condensation
	Vacuolization of the cytoplasm
	Formation of autophagic vacuoles
Necrosis	Cytoplasmic swelling
	Rupture of plasma membrane
	Swelling of cytoplasmic organelles
	Moderate chromatin condensation

Morphological characteristics represent the basis for discriminating between apoptosis, autophagy and necrosis. Various molecular events have been demonstrated in the different modes of cell death, but a set of biochemical critera that may be used to discriminate between them remains to be defined.

1.12 Mitochondria in cell death regulation

Mitochondria are highly specialized organelles consisting of an extensively folded inner membrane (IM), an intermembrane space (IMS) and an outer membrane (OM). The IM contains the mitochondrial respiratory chain consisting of four protein complexes involved in adenosine triphosphate (ATP) production through the process of oxidative phosphorylation. The IMS contains various cell death agonists, and the OM harbours proteins functioning as ion pumps and regulators of its stability. In addition to its prominent role in energy production, mitochondria play a central role in regulation of programmed cell death (91-93).

The stability of the OM is regulated by the Bcl-2 family of proteins, and mitochondrial OM permeabilization (MOMP) results in release of IMS cell death agonists to the cytoplasm (94). IMS proteins include caspase-activators like cytochrome c, Smac/DIABLO and Omi/HtrA2, and caspase-independent factors like apoptosis inducing factor (AIF) and endonuclease G (95). The IMS proteins have been shown to further promote cell death by antagonizing inhibitors of apoptosis proteins (IAPs). Cytochrome c is normally involved in mitochondrial ATP production, but when released into the cytosol it complexes with apoptosis protease-activating factor (Apaf-1), ATP and procaspase-9 to form the apoptosome. A simplified overview of mitochondria-mediated cell death is provided in Figure 4.

The exact molecular mechanism underlying MOMP is still not defined. An increase in permeability of the IM to solutes with molecular masses less than 1,500 Da has been shown to result in loss of mitochondrial membrane potential, mitochondrial swelling and rupture of the OM (96). This process is termed the mitochondrial permeability transition (MPT), and the mitochondrial permeability transition pore (PTP) has been suggested to play an important role (97).

Another mechanism by which mitochondria participate in cell death induction is via production of excess levels of intracellular ROS (91, 92). The mitochondrial respiratory chain contains several red-ox centres that leak electrons to molecular oxygen, resulting in generation of oxygen radicals. The cell converts oxygen radicals into hydrogen peroxide or other ROS, before elimination by cellular antioxidant systems including glutathione GSH and thioredoxins. ROS production by the mitochondrial respiratory chain has been shown to cause damage to proteins, DNA and mitochondrial membranes, resulting in MOMP and cell death (98).

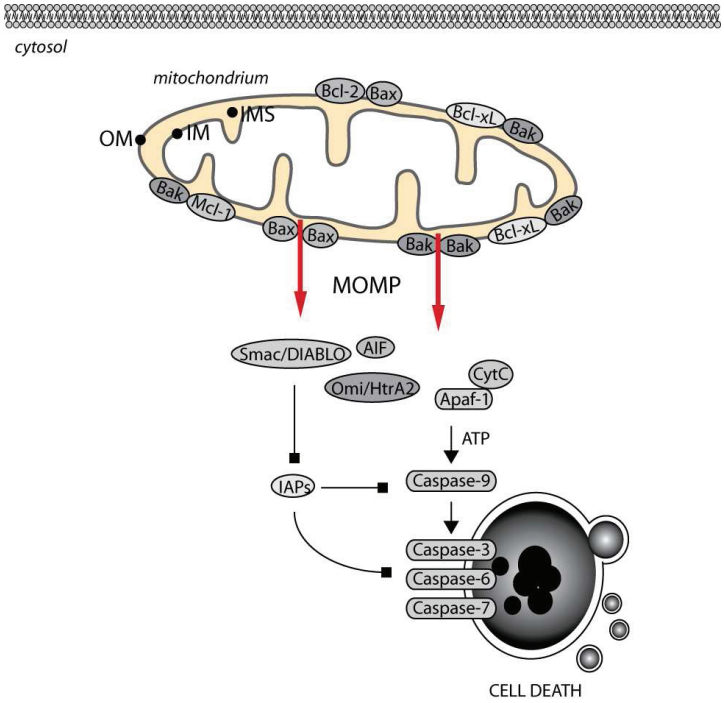

Figure 4. Simplified overview of mitochondria-mediated cell death

1.13 Bcl-2 family proteins and programmed cell death

The Bcl-2 family of proteins consists of anti-apoptotic and pro-apoptotic members involved in regulation of cell survival and death (94). The impact on mitochondrial OM stability and induction of MOMP represent the best studied processes involving Bcl-2 proteins.

The founding member of the Bcl-2 family is the anti-apoptotic Bcl-2 protein, which was discovered by characterization of genes involved in the 14;18 translocation found in follicular non-Hodgkin B-cell lymphoma (99). The Bcl-2 proteins contain 1 to 4 conserved Bcl-2 homology (BH) domains and are divided into: 1) anti-apoptotic proteins consisting of BH domains 1-4 (Bcl-2, Bcl-xL, Mcl-1, A1/Bfl-1), 2) pro-apoptotic proteins containing BH domains 1-3 (Bax, Bak, Bok/Mtd) and 3) pro-apoptotic proteins with only BH domain 3 (BH3-only proteins; e.g. BID, Bad, Noxa, PUMA)(94). The BH3 domain represents the region that allows interaction between Bcl-2 family members, and is also found in other proteins that interact with Bcl-2 proteins (100, 101).

The pro-apoptotic Bax and Bak proteins perturb mitochondrial integrity by forming homo-oligomers which function as pores in the OM (102, 103). Anti-apoptotic Bcl-2 family members antagonize Bax and Bak and stabilize the mitochondrial OM by forming heterodimers with the pro-apoptotic proteins (104). The BH3-only members favour pore-formation by Bax and Bak by antagonizing anti-apoptotic Bcl-2 proteins (105). If the balance between anti- and pro-apoptotic Bcl-2 family proteins is shifted towards the pro-apoptotic branch, the result is MOMP, which releases IMS death agonists into the cytosol (94). Bcl-2 family members have also been shown to associate with components of the PTP, which transverses both the mitochondrial IM and OM (97). Pro-apoptotic Bcl-2 family proteins like Bax, Bak and tBID have been suggested to induce pore opening by interacting with the voltage dependent anion channel (VDAC), whereas anti-apoptotic Bcl-xL induces its closure (106-108).

The anti-apoptotic Bcl-2 protein is frequently over-expressed is hematologic malignancies, including AML, where it confers chemotherapeutic resistance (109, 110). Bcl-2 and Bax levels have been shown to correlate with spontaneous apoptosis in AML cells *in vitro* (81), and the Bax/Bcl-2 ratio in *de novo* AML patients has been reported to predict clinical response and outcome (79). Targeting of anti-apoptotic Bcl-2 family members, like Bcl-2 and Bcl-xL, represents a promising therapeutic strategy where different approaches are being explored. A range of small molecule inhibitors of anti-apoptotic Bcl-2 proteins has been identified by screening of natural compounds and rational design techniques (111). Examples of potent inhibitors include Gossypol, Apogossypol and HA14-1 (112-114). Bcl-2 over-expression antagonizes both apoptosis and necrosis, and it has been shown to inhibit Beclin-1-dependent autophagy when localized in the ER (115). It has been suggested that Bcl-2 suppresses autophagy to levels that are compatible with cell survival rather than cell death (116).

The anti-apoptotic Mcl-1 protein has also been found to be over-expressed in cancers, mediating resistance to cell death-induction (117-119). Mcl-1 degradation is required for MOMP following UV irradiation (120) and therapeutic targeting of Bcl-2 family members has been shown to depend on Mcl-1 neutralization (121). Anti-apoptotic Mcl-1 has also been demonstrated to bind Beclin-1, which contains a BH3-like domain (101, 122).

1.14 Induction of death via cell surface receptors

Cell death may be induced by signals from within the cell (e.g. DNA damage, hypoxia, ROS) or by activation of cell surface death receptors of the tumor necrosis factor (TNF) superfamily of receptors. Binding of extracellular death ligands result in receptor-oligomerization and recruitment of intracellular adaptor proteins and procaspase-8. Together these components constitute the death inducing signaling complex (DISC), where procaspase-8 becomes activated and mediates cleavage-activation of down-stream effector caspases (82). Additionally, caspase-8 cleaves the BH3-only pro-apoptotic Bcl-2 family member BID to truncated (t)BID, generating the activate version of this cell death agonist (123). tBID promotes mitochondrial cytochrome c release and connects the receptor-mediated and the mitochondria-mediated cell death pathways (123, 124). The anti-apoptotic Mcl-1 protein has been shown to interact with and counteract the death inducing activity of tBID (125).

Cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (c-FLIP), a catalytic inactive caspase-8 homologue, has been shown to antagonize receptor-mediated cell death at the DISC (126, 127). FLIP primarily localizes to the cytosol and is expressed as three isoforms, c-FLIP$_s$ and c-FLIP$_R$, representing two short forms and c-FLIP$_L$, which is a full length version. Most reports have demonstrated that c-FLIP$_L$ inhibits receptor-mediated death, but a potential role in the NK-κB survival pathway has been suggested (128). A simplified overview of receptor-mediated cell death is provided in Figure 5.

c-FLIP over-expression provides resistance to receptor-mediated apoptosis in B cell chronic lymphocytic leukemia, multiple carcinomas (e.g. colourectal, gastric, pancreatic and ovarian) and Hodgkin/Reed-Sternberg cells (129-131). Down-regulation of c-FLIP levels sensitizes tumour cells to apoptosis-induction by extracellular death ligands (132-134). Various anti-cancer drugs including doxorubicin, actinomycin D, cycloheximide (CHX) camptothecin (CPT) and bortezomib have been shown to mediate reduced c-FLIP levels (129, 135). Phenoxodiol, a synthetic analogue of a soybean isoflavone, has been reported to inhibit XIAP and c-FLIP, and represents a potential anti-cancer therapeutics (136, 137).

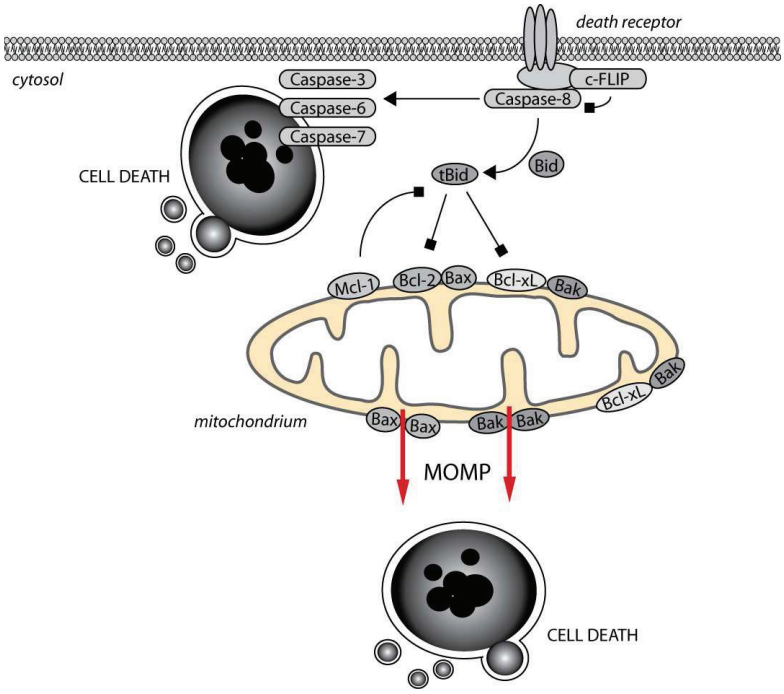

Figure 5. Simplified overview of receptor-mediated cell death and its relation to the mitochondria-mediated pathway.

1.15 p53 - stress sensor and tumour suppressor

The p53 protein is a sequence-specific transcription factor that can halt progression through the cell cyle or initiate cell death in response to DNA-damage and other types of cellular stress (138). p53 has a prominent role as a tumour suppressor and is found mutated and inactivated in over 50% of human cancers (139-141). However, p53 is most often wild type (wt) in hematological malignancies, and less than 10% of AML patients carry p53 mutations at the time of diagnosis (142, 143). The prevalence of p53 mutations is found to be higher in secondary AML and increases with patient age (139).

p53 has a short half-life in non-stressed cells due to its interaction with the human double minute protein (Mdm2), which is a p53-specific E3 ubiquitin ligase (144, 145). Mdm2-mediated mono-ubiquitination of lysine residues precedes poly-ubiquitination by p300, which targets p53 for proteasomal degradation (146-148). Stress signals, including DNA-damage, hypoxia and nutrient deprivation, induce various post-translational

modifications (PTMs) of p53 affecting its stability, localization and function. p53 contains multiple lysine, threonine and serine residues that can accept various modifications, which include ubiquitination, phosphorylation, acetylation and sumoylation (138). Phosphorylation of serine 15 and 37 in the N-terminal domain is detected rapidly after stress induction, and has been shown to inhibit Mdm2-mediated degradation, resulting in p53 stabilization and activation (149). Acetylation of lysine residues in the C-terminal domain also inhibits the interaction with Mdm2, and has been shown to enhance p53 transcriptional activity, as well as to be crucial for its transcription-independent functions (150, 151).

When acting as a tumour suppressor, the p53 protein may mediate programmed cell death via both transcription-dependent and transcription-independent mechanisms. p53 has a well known function as a sequence-specific transcription factor that induces expression of pro-apoptotic genes of the Bcl-2 family (152-154). The p53-inducible Bax, Noxa and PUMA proteins promote MOMP and mitochondria-mediated cell death. In addition, p53 has been shown to induce other mitochondrial proteins that favour MOMP through oxidative reactions, like ferredoxin reductase and proline oxidase (155, 156). Furthermore, p53 has been shown to induce expression of the cell surface death receptor 5 (DR5) following DNA damage (157).

Stress stimuli have been shown to trigger rapid trafficking of p53 to the mitochondria, an effect preceding its nuclear accumulation (158, 159). The mitochondrial trans-localization was demonstrated to be induced by Mdm2-mediated mono-ubiquitination and not by phosphorylation or acetylation (160, 161). In the OM membrane p53 interacts with anti-apoptotic Bcl-2 proteins and inhibits their stabilizing function (159, 162). p53 disrupts the complex between Bak and Mcl-1, and has been shown to mediate Bak oligomerization (163). p53 has also been shown to activate Bax, promoting MOMP and cell death induction (164). In addition to direct inhibition of the stabilizing function of anti-apoptotic Bcl-2 family proteins, p53 may repress transcription of these and other cell death antagonists like the IAP protein Survivin (165, 166). Another transcription-independent mechanism by which p53 promotes cell death is by mediating cell surface trafficking of the TNF receptor Fas (CD95) from cytoplasmic stores (167).

Cancer cells expressing wt p53 still have the potential to evade induction of apoptosis. This is due to the presence of other mutations opposing the death-inducing activity of p53. Different mechanisms have been demonstrated and include over-expression of Bcl-2, Mdm2 and the human papilloma virus E6 protein, loss of the Mdm2 inhibitor $p14^{ARF}$ and

aberrant modulation of p53 by kinases/phosphatases (168, 169). AKT has been reported to contribute to chemoresistance by attenuating p53 phosphorylation, which will inhibit its stabilization and accumulation (170). *MLL* translocations represent a poor prognostic factor in AML, and a proposed mechanism for MLL fusion proteins involves reduced p53 acetylation and stabilization (171, 172).

Activation of p53 and induction of cell death represents a therapeutic target in cancers, which may be triggered in response to radiation- and chemotherapy (139, 173). Novel non-genotoxic strategies for p53-activation are currently being investigated, examples include the Nutlins which are small molecular antagonists of Mdm2-mediated degradation of wt p53 (174, 175). Another promising compound is PRIMA-1 which restores the tumour suppressing function of mutated p53 (176). Several drugs that target the Bcl-2 protein promote MOMP and cell death through mechanisms involving activation of p53 (177).

2. AIMS OF THE STUDY

An organic extract of the natural stimulant khat was previously shown to induce programmed cell death in AML cell lines, while being less cytotoxic to normal PBMCs. Few studies have aimed at evaluating cellular and molecular events underlying khat toxicity, compared khat with a characterized drug or evaluated the effects of khat and known constituents on normal immune cells.

This study was undertaken to:

- elucidate cellular and molecular effects underlying khat-induced cell death in AML cell lines in comparison to a well characterized cancer therapeutics
- investigate early cellular and molecular effects of khat and the khat amphetamines in normal peripheral immune cells
- identify the compound(s) mediating the cytotoxic effects of the khat extract

3. METHODOLOGICAL CONSIDERATIONS

3.1 Preparation of an organic khat extract

Various organic solvents have been used for khat extraction in studies of *in vitro* effects of khat. When preparing an extract of khat we used methanol extraction as in the previous studies on khat cytotoxicity in AML cell lines (62, 63). The protocol used had been shown to produce an extract with appreciable levels of the natural khat amphetamines: cathinone, cathine and norephedrine (62). In short, the khat material was extracted with pure methanol, the solvents evaporated and the remaining semi-solid residue dissolved in dimethylsulfoxid (DMSO) at 0.2 g/ml for storage at -80^{o}C.

In this study, two different batches of khat from the Meru district in Kenya were extracted and used in experiments. The khat grown in Meru is known to be cultivated in the traditional way and has not been treated with pesticides. However, there is an ongoing debate whether the use of such chemicals should be introduced in Meru (personal communication, O.M. Lukandu and S. Kimani; European Science Foundation conference, Linköping, Sweden, 2009). Khat farmers in Yemen are using pesticides and there are rising concerns about potential adverse health effects derived from its use (57, 178).

The first khat batch was harvested and processed in February/March 2005, and the extract was used in the experiments in paper I and IV. The second khat batch was harvested and processed in September/October 2007, and used in experiments in paper II and III. When comparing the toxic potentials of the two extracts they were observed to induce similar levels of cell death in AML cell lines. However, the extract from 2005 appeared slightly more toxic than the extract from 2007 (results not shown). The chromatograms of the two extracts displayed similar profiles, indicating a similar chemical composition and a standardized extraction procedure (Figure 6). The compositions of the extracts were not expected to be identical, since factors like season and variations in growth conditions will influence the chemical profile (7).

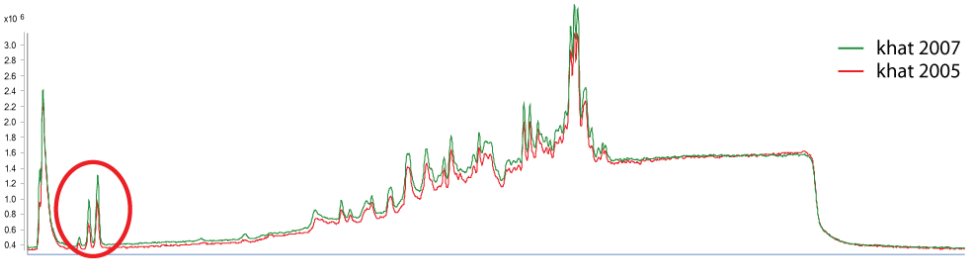

Figure 6. Chromatograms of the khat extracts from 2005 and 2007. The circle indicates elution of the khat amphetamines: norephedrine, cathine and cathinone. The figure is based on HPLC analyses performed by K. O. Fossan.

3.2 Experimental concentrations of khat and its amphetamines

The natural khat amphetamines, and in particular cathinone, represent the most well studied constituents of khat. The studies have elucidated the chemistry and pharmacology of the khat amphetamines, and dealt with methods for their detection in blood plasma, urine and hair (15, 16, 18, 19).

In this study, AML cell lines and normal peripheral leukocytes/lymphocytes were exposed to various dilutions of the khat extracts. The experimental dilution designated 200 µg/ml in paper I is based on the weight of semi-solid khat material dissolved in DMSO (0.2 g/ml). The 200 µg/ml designation hence represents a 1:1000 dilution of the DMSO-solution of the khat extract. This designation does not provide information about the actual concentrations of khat constituents, and the concentrations of the khat amphetamines were therefore determined using high pressure liquid chromatography and tandem mass spectrometry (HPLC-MS-MS). Table 5 provides an overview of the concentrations and molarities of the khat amphetamines in the khat extracts and in experimental cell cultures.

Cathinone is relatively unstable and is transformed to cathine upon wilting of khat leaves, while being metabolized predominantly to norephedrine *in vivo* (9, 16, 17). Cathinone is therefore suited as a reference substance, indicating the freshness of the extracted khat batch and the stability of the extracted material.

Table 5. Overview of the concentrations and molarities of the khat amphetamines in the two khat extracts and experimental dilutions used in this study.

Khat extract	Cathinone (mw 150.1)		Cathine (mw 152.1)		Norephedrine (mw 152.1)	
	mg/ml	M	mg/ml	M	mg/ml	M
2005						
Stock solution	1.5	0.01	1.4	9.2×10^{-3}	0.2	1.3×10^{-3}
10^{-3} dilution	1.5×10^{-3}	1.0×10^{-5}	1.4×10^{-3}	9.2×10^{-6}	2.0×10^{-4}	1.3×10^{-6}
2007						
Stock solution	2.5	0,017	3.0	0.02	0.3	2.0×10^{-3}
10^{-3} dilution	2.5×10^{-3}	1.7×10^{-5}	3.0×10^{-3}	2.0×10^{-5}	3.0×10^{-4}	2.0×10^{-6}
3.16×10^{-4} dilution	7.9×10^{-4}	5.4×10^{-6}	9.5×10^{-4}	6.3×10^{-6}	9.5×10^{-5}	6.3×10^{-7}
10^{-4} dilution	2.5×10^{-4}	1.7×10^{-6}	3.0×10^{-4}	2.0×10^{-6}	3.0×10^{-5}	2.0×10^{-7}

Maximal serum concentrations following khat chewing have been measured to be, in µg/l, 58.9 ± 18.8 for cathinone, 71.2 ± 13.9 for cathine and 72.1 ± 12.2 for norephedrine (15). Each of the measured serum values correspond to concentrations in the range of 10^{-7} M. It should be noted that the amount of khat chewed by the study participants was one quarter of the amount used by an average chewer, and that the amount chewed varies widely (15). Due to the efficient extraction of the khat amphetamines into saliva, cells and tissues in the oral cavity are exposed to relatively high concentrations of the khat amphetamines (15). Previous estimates of cathinone concentrations in saliva suggest that the concentrations of khat amphetamines used in this study are within the range of concentrations found in the oral cavity (59).

3.3 AML cell lines and normal peripheral blood leukocytes

There are many advantages of using cell lines in studies of toxicity and in pre-clinical evaluation of cellular and molecular effects by potential drugs. These include ease of cultivation, commercial availability, genetic and phenotypic characterization and the possibility of comparing results with previous *in vitro* studies. However, several problems are associated with the use of cancer cell lines, like recurrent mycoplasma infections, cell line mix-ups and the differences in genetic expression between the cell line and the cancer it represents. For instance, most available AML cell lines carry a mutated *TP53* gene, which contrasts with the disease where p53 is reported to be wt in 90% of the patients (142, 143). However, the leukemic cell lines MOLM-13 and MV-4-11 express wt p53 (179, 180), and

were therefore used in paper I and II, where khat-induced cellular and molecular events were evaluated. Further, the difference these cell lines exhibited in khat sensitivity made them valuable when studying the mechanisms underlying khat toxicity.

The p53 protein was studied in paper II, and in order to establish the functional role of p53 activation in khat-mediated cell death, we tested different cell systems with deleted or reduced levels of p53. Experiments were performed with bone marrow cells from p53 -/- mice and their wild-type littermates (181), and we also used MOLM-13 wt and MOLM-13 shp53 cells, where the expression level of p53 had been reduced by the introduction of short hairpin (sh) RNAs against p53 (182).

In paper III we investigated early effects by khat and the khat amphetamines on intracellular signaling in normal peripheral leukocytes. The use of primary cells enabled us to study effects on healthy cells, and in addition provided us with the opportunity to compare effects in subsets of a complex population of immune cells. The study provided information about effects on normal cells, but further studies using multi-parameter flow cytometric analysis could shed light on potential theraperutic effects by the amphetamines, which were shown to attenuate activating protein modifications (4).

3.4 Determination of cell death, viability and proliferation

Induction of cell death in AML cell lines was determined based on fluorescence microscopy of cells that had been fixed and stained with the DNA-intercalating fluorochrome Hoechst (183). Normal nuclei displayed a diffuse nuclear staining, whereas non-normal nuclei appeared more intensely stained, with or without the nuclear fragmentation characteristic of apoptotic cells (85). This method is well established as a routine assay for determination of cell death. Ultra structural cellular features were evaluated with transmission electron microscopy (TEM), representing a principal method used to distinguish between different modes of cell death (85, 90). In paper I, TEM indicated khat-mediated activation of autophagy in MOLM-13 cells, with formation of autophagosomes and cytosolic vacuolization. In paper II, we stained khat-treated MOLM-13 cells with Lyso Tracker® Red DND-99, demonstrating increased levels of acidic cellular organelles, further suggesting involvement of the autophagosomal-lysosomal pathway (184). Compared to AML cell lines, it is difficult to evaluate nuclear morphology in bone marrow cells and PBMCs following

Hoechst-staining. In paper II and III, assessment of cell death in primary cells was therefore based on flow cytometric analyses following Annexin-FITC/PI staining.

Cell viability/proliferation of AML cell lines was evaluated with the WST-1 assay. The assay relies on the activity of complex II in the mitochondrial respiratory chain, which converts the WST-1 salt (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt) to soluble formazan dye, which can be quantified by measuring absorbance (185). The presence of functional mitochondria will therefore provide a means of assessing viable and proliferating cells. High-resolution respirometry, which measures oxygen consumption by metabolic active mitochondria, was used to complement the results based on nuclear morphology, the WST-1 assay and TEM in paper I. The results demonstrated that by using different methods to evaluate cytotoxicity, it was possible to elucidate the underlying mechanisms of khat-induced cell death. When assessing degree of PBMCs proliferation in paper III, we used a ^{3}H-thymidine incorporation protocol based on the whole blood assay described by Wendelbo *et al.* (186, 187). Unfortunately, we did not assess the amount of cell death simultaneously as we determined degree of ^{3}H-thymidine incorporation. However, khat was shown to induce cell death after 6 hours of exposure based on Annexin-FITC/PI staining, and the reduced proliferation of PBMCs seen after 4 days was probably in part due to induction of cell death in a fraction or subset of the cells.

In order to evaluate the cytotoxic potential of khat and underlying mechanisms, khat was compared side-by-side with camptothecin (CPT) in paper I and II. CPT is an alkaloid that originally was isolated from the Chinese plant Camptotheca acuminata (188). CPT induces apoptosis in various cancer cells and its derivatives irinotecan and topotecan are currently used as anti-cancer therapeutics (188). CPT mediates topoisomerase I-linked DNA breaks by preventing religation and has been shown to induce p53, Noxa and Mcl-1 (189, 190). CPT and its analogues are collectively termed camptothecins, and represent one of the four main classes of anti-cancer compounds from plants (1).

3.5 Determination of protein levels and signaling events

Protein levels in AML cell lines were determined primarily using one-dimensional polyacrylamide gel electrophoresis (1D-PAGE) and Western blotting in paper I and II. The KODAK Image Station 2000R software was used for quantification of Western blots and determination of fold change in protein levels. In paper I, where we assessed the impact of

khat-treatment on procaspase-8 activation, the Western blot analyses were complemented with the use of a colourimetric assay to verify cleavage and enzymatic activation.

When studying the p53 protein in paper II, we used two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) in addition to 1D-PAGE. This technique is superior to 1D-PAGE when studying expression of various p53 isoforms and levels of PTMs (191, 192). When analyzing the 2D images, we used an analysis method that was developed to enable correlation of separate analyses and biological variables (193). p53 PTMs were in addition analyzed with modification-specific antibodies and flow cytometric analyses as previously described (4). The results demonstrated that flow cytometric analyses of the different p53 modifications represented a more sensitive technique compared to the 1D-PAGE protein analyses.

Modification-specific antibodies and flow cytometric analyses were also used in paper III, when we analysed intracellular signaling events in normal peripheral leukocytes. A major advantage of this technique over Western blotting when studying proteins is that it enables multi-parameter analyses of intracellular signaling events in complex cell populations. It was therefore the natural choice of method when we assessed signaling events in subsets of the complex population of immune cells. The many advantages and also the challenges associated with this technique have been reviewed by Krutzik *et al.* (194).

4. SUMMARY OF PAPERS

Paper I

Camptothecin and khat (*Catha edulis* Forsk.) induced distinct cell death phenotypes involving modulation of c-FLIP$_L$, Mcl-1, procaspase-8 and mitochondrial function in acute myeloid leukemia cell lines

Cellular and molecular effects of khat were investigated using AML cell lines with diverse genetic characteristics. The cytotoxic effects of khat were compared with the cancer therapeutics camptothecin (CPT), in order to evaluate the toxic potential of khat and to elucidate the mechanisms underlying toxicity. The AML cell lines demonstrated varying sensitivities to khat, where some cell lines were shown to be sensitive to khat-induced cell death whereas others appeared resistant. The different sensitivities could not be correlated to endogenous Bcl-2 and Bax levels, or to selected prognostic factors (*FLT3* and *TP53*). Khat treatment had a profound effect on mitochondrial structure and function in MOLM-13, in contrast to CPT. Khat was observed to induce formation of autophagosomes and cytosolic vacuolization in MOLM-13 cells, suggesting activation of autophagy. Khat mediated reduced levels of anti-apoptotic Mcl-1 protein in MOLM-13, whereas both khat and CPT were shown to activate procaspase-8 and induce cleavage of its endogenous antagonist c-FLIP$_L$.

Paper II

Modulation of p53 isoforms and its post-translational modifications reflect the cytotoxicity of a botanical khat extract

The effects of khat-treatment on induction of p53 PTMs, on p53 isoform distribution and the contribution of p53 in khat-mediated cell death were evaluated. The analyses included the khat-sensitive MOLM-13 cell line and the khat-resistant MV-4-11 cell line. p53 was subjected to phosphorylations and acetylation in both MOLM-13 and MV-4-11 cells, but its accumulation was only demonstrated in MOLM-13. When comparing khat-mediated toxicity in bone marrow cells from p53 -/- mice with cells from normal mice, no protective effect against khat was observed. Similarly, MOLM-13 cells transduced with shp53 were not more resistant to khat-mediated cell death compared to wt MOLM-13 cells. Treatment of the khat resistant MV-4-11 cell line with p38 inhibitors increased its sensitivity to khat. Pre-treatment

of MOLM-13 cells with p38 inhibitors abolished khat-induced activation of autophagy, whereas this pathway was unaffected in MV-4-11. The study indicated that khat acted independently of p53, but suggested that p53 isoform pattern and PTMs could be used to in identification and characterization of chemical probes and potential anti-cancer therapeutics.

Paper III

Natural khat-derived amphetamines attenuate phosphorylation of AKT, STAT6, CREB and p53 in peripheral leukocytes

Multi-parameter flow cytometric analyses of signaling proteins were used to investigate early responses in normal periperhal leukocytes to treatment with khat and its natural amphetamines: cathinone, cathine and norephedrine. The leukocytes were labeled with modification-specific antibodies against stress sensor proteins and signal transducers of various intracellular pathways (AKT, CREB, ERK1/2, p38, STAT1/3/5/6 and p53). The different subsets of the leukocytes: T-lymphocytes, B-lymphocytes/natural killer (NK) cells and the myeloid population (neutrophile granulocytes and monocytes) responded differently to the various treatments. The khat extract was generally observed to mediate activation of signaling proteins, detected as increased levels of phosphorylation and acetylation, whereas the khat amphetamines attenuated activating modifications. Khat resulted in increased p53 and phosphorylated p38, and was observed to induce cell death and reduced proliferation of PBMCs, whereas the khat amphetamines appeared to have a stimulating effect on cell division.

Paper IV

Bio-guided isolation of a major cytotoxic constituent in khat (*Catha edulis* Forsk.) unrelated to natural khat amphetamines

In order to identify the cytotoxic constituent(s) in the khat extract, the extract was subjected to high pressure liquid chromatography (HPLC), and the resulting fractions screened for toxicity in the khat-sensitive cell lines HL-60 and MOLM-13. The cell-based screen resulted in the identification of three separate cytotoxic fractions, mediating reduced viability/proliferation and induction of cell death. Both cytotoxic and neutral fractions were subjected to mass spectrometry analyses in order to identify the main cytotoxic component(s). The presence of the khat amphetamines did not correlate with the cytotoxicity

of the khat fractions, and treatment of HL-60 and MOLM-13 with cathinone, cathine and norephedrine further suggested that these constituents were relatively non-toxic. The most cytotoxic khat fraction contained a molecule with a mass of 1452 Da, and the MS fragmentation pattern suggested that the molecule consisted of two central phenylpropanoid glycosides with five attached comaryl-groups. This study is the first to describe a cytotoxic phenylpropanoid glycoside in an extract of khat.

5. GENERAL DISCUSSION

5.1 Specificity in cell death-induction and genetic aberrations

Khat was demonstrated to mediate cell death in a subset of genetically diverse AML cell lines, in paper I and II. The observations of AML cell lines to be either sensitive or resistant to khat suggested involvement of specific death pathways. In contrast to khat, the anti-cancer therapeutics CPT was observed to induce cell death in all cell lines tested, although with varying efficacies. The specificity in khat-mediated cell death has been further demonstrated in preliminary experiments using primary cells from AML patients. The experiments showed that khat caused cytotoxic effects in leukemic cells from 1 out of 4 patients (results not shown).

The AML cell lines used in this study are characterized by various genetic aberrations that could explain the differences in sensitivity. In paper I we concluded that neither the presence of *FLT3*-ITDs nor the mutational status of *TP53*, both important prognostic markers, seemed to account for the different khat sensitivities exhibited by the cell lines. However, the presence of other genetic aberrations may influence the signaling pathways regulated by the Flt3 receptor and the tumour suppressor p53. For instance, *MLL* translocations are found in leukemias of both myeloid and lymphoid origin and represent a poor prognostic factor with adverse effects on treatment response (195). A proposed mechanism for MLL fusion proteins involves reduced acetylation of p53 by p300, which would abrogate its stabilization and activation (172).

The khat-resistant MV-4-11 cell line carries the t(4;11) translocation, the most common *MLL* translocation in acute lymphoblastic leukemia, encoding the MLL-AF4 fusion protein. However, the khat-sensitive MOLM-13 cell line has been shown to expresses a MLL-AF9 fusion mRNA, resulting from a minute chromosomal insertion, ins(11;9)(q23;p22p23)(196). The expression of distinct MLL fusion proteins in MV-4-11 and MOLM-13 could indicate different effects on intracellular signaling. However, the presence of chimeric MLL proteins in both cell lines could also suggest other characteristics as more important in determining the differences in khat sensitivity (197).

MV-4-11 has been shown to carry a *K-RAS* mutation which has been suggested to inhibit the function of the p53 protein (198, 199). Oncogenic K-Ras has also been shown to activate the PI3K/AKT pathway, which could explain the lack of Mcl-1 down-regulation in this cell line as compared to MOLM-13 in paper I (200). Further, oncogenic K-Ras could mediate increased levels of ROS and constitutive activation of p38, which acts up-stream of p53 (201, 202). In conclusion, the AML cell lines used in this study carry a range of genetic aberrations which could provide the molecular basis for the different khat sensitivites that were observed.

In paper III, we observed that khat was less cytotoxic to normal PBMCs compared to the khat-sensitive AML cell lines, in agreement with previous results (62). Khat was shown to induce significant cytotoxic effects after 6 hours, and cell proliferation was observed to be inhibited by approximately 50% after 4 days of treatment. Despite the induction of cell death, the study demonstrated the presence of viable and actively proliferating cells after long-time exposures to khat. However, the study did not assess cytotoxic effects in the various subsets of the complex population of immune cells. The treatments were observed to induce different intracellular responses in the cell subsets, and it is reasonable to expect that the populations would exhibit different sensitivities to khat.

5.2 Modes of programmed cell death

Mitochondria are known to play a central role in induction of different types of cell death, and may mediate death through release of cell death agonists and by generating excess levels of ROS (92). When comparing ultra structural features by TEM in paper I it was evident that khat, in contrast to CPT, caused extensive damage to the mitochondria in MOLM-13. Pharmacological targeting of mitochondrial function has been suggested as a possible therapeutic intervention (203, 204). However, even cells in late phases of cell death, contained apparently intact mitochondria in addition to the damaged ones. This suggested, although impaired, ongoing mitochondrial ATP production which could fuel the energy-dependent processes of apoptosis and autophagy (184). Impaired mitochondrial function may trigger autophagy, as it is initiated in times of nutrient deprivation and other forms of cellular stress (87, 88). Therefore, mitochondrial impairment could have triggered the morphological characteristics of autophagy that were observed in khat-treated MOLM-13 cells in paper I.

Autophagosome formation was observed in cells in early stages of cell death, whereas extensive vacuolization of the cytoplasm characterized cells in late phases (85, 184).

In paper II, the use of Lyso Tracker® Red DND-99 demonstrated an increase in the level of acidic cellular organelles, further indicating activation of the autophagosomal-lysosomal pathway in MOLM-13 (184). When pre-treating MOLM-13 with specific p38 inhibitors, the khat-induced increase in acidic organelles was abolished. p38 acts up-stream of p53 and the inhibitors could therefore have hindered p53 activation (201). p53 has the potential to trigger autophagy, but is reported to play a dual role, with nuclear p53 inducing autophagy genes, whereas cytoplasmic p53 represses its activation (205). However, p38 has been shown to induce transcription of autophagy genes in response to elevated ROS levels, indicating that khat-mediated autophagy could be induced up-stream of p53 (206). In order to further verify the involvement of autophagy, biochemical events like dissociation of Beclin-1 from anti-apoptotic Bcl-2 family members and degradation of the p62 protein should be investigated (85).

We previously reported that khat-mediated cell death in HL-60 displayed typical morphological and biochemical characteristics of apoptosis, including nuclear fragmentation and procaspase-3 activation (62). When comparing the nuclei in cells undergoing khat-mediated death, MOLM-13 and HL-60 were observed to display different nuclear morphologies (results not shown). Whereas chromatin was observed to be condensed and localized to the margins of the nuclei in MOLM-13, HL-60 demonstrated characteristic apoptotic nuclei with condensed and fragmented chromatin (62). Autophagy as a mode of cell death is characterized by absence of chromatin condensation, and this observation indicated that several death-inducing pathways could be activated in MOLM-13. Further, the observation of distinct nuclear morphologies indicated that khat triggered different modes of programmed cell death in individual AML cell lines. The morphological differences were further supported by Western blot analyses of khat-treated MOLM-13 cells, which demonstrated limited procaspase-3 activation (results not shown), contrasting with the analyses of HL-60.

It has been demonstrated that the concentration of anti-cancer drugs may dictate the mode of cell death in experimental cell cultures. Drugs may induce necrosis when administered at a high dose while inducing apoptosis at lower concentrations (90, 184). It is therefore possible that the type of programmed cell death and the exact signaling pathway(s) involved could be altered by changing the concentration of khat. It has also been

demonstrated that dying cells may display morphological characteristics of more than one type of cell death (85, 90).

5.3 Potential molecular targets of khat and the khat amphetamines

In paper I we focused on the Bcl-2 family of proteins, important regulators of mitochondria-mediated cell death (94). Bcl-2 family proteins are frequently aberrantly expressed in cancer, and over-expression of anti-apoptotic members is shown to mediate therapeutic resistance and poor survival (109, 110). The level of khat-induced cell death was reduced by increased Bcl-2 in the rat acute myeloid leukemia IPC-81 Bcl-2 cell line. This cell clone is transfected to express a *BCL-2* gene of human origin and has been shown to be resistant to pro-apoptotic stimuli (207). In agreement with previous reports, CPT-induced cell death was inhibited in this cell line (208, 209). Khat-mediated cell death was significantly inhibited by Bcl-2 over-expression in the human promyelocytic NB4 cell line. However, there did not appear to be a correlation between khat sensitivity and the endogenous levels of Bcl-2 and Bax in the various AML cell lines. Furthermore, Western blot analyses of khat-treated cells demonstrated that the protein levels of Bcl-2 and Bax remained relatively constant. These observations suggested that sensitivity was dictated by other molecular characteristics and that khat did not target the Bcl-2/Bax proteins directly.

However, khat was shown to mediate significant reductions in expression levels of the anti-apoptotic Mcl-1 protein. This Bcl-2 family protein primarily exerts its function in the mitochondrial OM where it binds and antagonizes pro-apoptotic Bcl-2 family members. Mcl-1 degradation following UV irradiation has been shown to be required for mitochondrial cytochrome c release and subsequent activation of procaspases (120). It has been reported that therapeutic targeting of Bcl-2 family members and induction of apoptosis depends on neutralization of Mcl-1 (121). In addition to its role in mitochondria, Mcl-1 has been shown to antagonize receptor-mediated cell death by inhibiting truncated (t) BID. tBID is generated by caspase-8 mediated cleavage of BID, and the truncated protein promotes cytochrome c release from mitochondria (123-125). Mcl-1 has also been reported to interact with Beclin-1, an autophagy-promoting protein that contains a BH3 domain (101, 122). BH3-only pro-apoptotic proteins of the Bcl-2 family promote autophagy by disrupting the binding of Beclin-1 to anti-apoptotic Bcl-2 proteins (210). Khat-mediated down-regulation of Mcl-1 could therefore be involved in induction of autophagy in MOLM-13.

The observations in this study indicate that Mcl-1 may represent a central molecular target in khat-induced cell death. Mcl-1 is frequently over-expressed in cancers and has been shown to mediate chemoresistance (117-119). The plant-derived compound silvestrol was recently shown to induce early reductions in Mcl-1 levels due to translational inhibition, mediating subsequent mitochondrial damage (211). Other plant-derived compounds, like Apogossypol, have also been demonstrated to target Mcl-1 and other anti-apoptotic Bcl-2 family members when inducing apoptosis (112, 113).

Pre-treatment of HL-60 with the specific caspase-8 inhibitor, Z-IETD-FMK, was previously shown to protect against khat-induced apoptosis (62). In paper I we demonstrated the presence of active caspase-8 in khat-exposed cells, further supporting involvement of signaling via cell surface death receptors. In addition, we demonstrated a relatively rapid cleavage of the caspase-8 antagonist c-$FLIP_L$ to the cleavage product p43-FLIP. This cleavage product has been shown to be generated by activated caspase-8, whereas procaspase-8 cleaves $FLIP_L$ to a fragment of 22 kDa, p22-FLIP. The smaller c-FLIP fragment has been shown to activate NF-kB signaling, hence mediating cell survival (128).

c-FLIP over-expression has been shown to confer resistance to receptor-mediated apoptosis in B-cell chronic lymphocytic leukemia, multiple carcinomas (e.g. colourectal, gastric, pancreatic and ovarian) and Hodgkin/Reed-Sternberg cells (129-131). Down-regulation of c-FLIP sensitizes tumour cells to apoptosis-induction by extracellular death ligands (132-134). Various anti-cancer drugs including doxorubicin, actinomycin D, CHX, CPT, bortezomib, cisplatin and trichostatin A have been shown to reduce c-FLIP levels (129, 135). The anti-cancer therapeutics phenoxodiol, an analogue of a soybean isoflavone, is believed to inhibit XIAP and c-FLIP, thereby facilitating induction of apoptosis (136, 137). The potential of c-FLIP as a therapeutic target has recently been reviewed (129, 212).

c-FLIP down-regulation has been shown to occur in conjunction with ROS generation (213), and c-FLIP cleavage was reported to induce ROS (214). Lukandu *et al*. demonstrated that khat-mediated apoptosis in oral keratinocytes and fibroblasts involved generation of ROS (60). These observations could suggest that caspase-8 mediates c-$FLIP_L$ cleavage in MOLM-13, which then could trigger increased levels of ROS. However, whether ROS may trigger c-FLIP down-regulation or if reduced c-FLIP levels may cause elevated levels of ROS, remains to be further elucidated. Interestingly, it has been shown that treating leukemic cells with inhibitors of ROS elimination and complex I inhibitors, mediates increased

sensitivity to drug-induced apoptosis (203). An overview of the results discussed in this section is provided in figure 7.

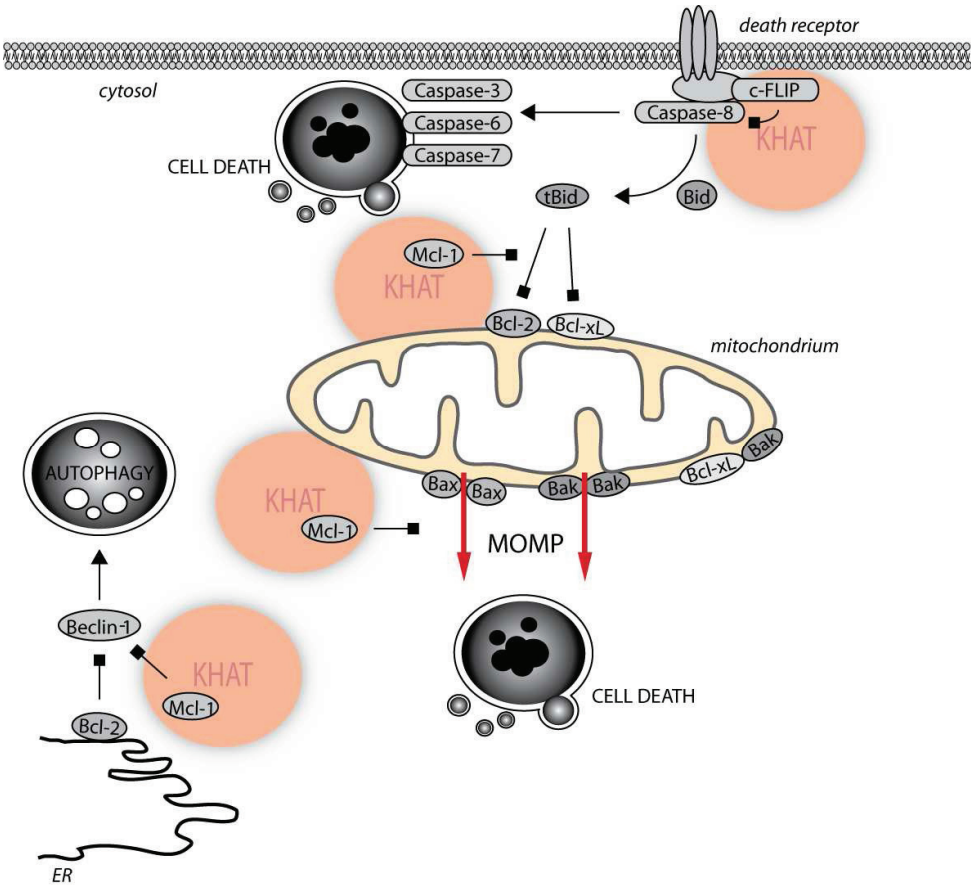

Figure 7. Overview of proteins that were affected by khat treatment and possible involved cell death pathways.

In paper III we compared single cell signal responses in normal peripheral blood leukocytes to khat and the khat amphetamines: cathinone, cathine and norephedrine. Whereas khat was observed to induce activating protein modifications, the khat amphetamines attenuated phosphorylation of several signaling transducers including AKT, STAT6 and CREB. This suggested that the khat amphetamines could have potential as inhibitors of signal transduction pathways. In a recent study by Krutzik *et al*. primary splenocytes were used to evaluate inhibitors of intracellular pathways first identified in a

leukemic cell line. The use of heterogenous population of immune cells demonstrated pathway-specificity and in addition identified cell type-specific inhibitors (4).

5.4 Modulations of the p53 protein and cytotoxic effects

We previously reported that khat activated p53 in primary oral keratinocytes and fibroblasts, mediating transcription of p21 and induction of cell cycle arrest (59). When exposed to a higher concentration of khat the oral fibroblasts and keratinocytes underwent programmed cell death, involving ROS and impaired mitochondrial integrity and function (60, 61). This indicated that p53 could be involved in khat-mediated cell death in AML cell lines, and regulation of the p53 protein was studied in paper II. However, we previously reported that AML cell lines without *TP53* and mutated *TP53* were sensitive to khat-induced cell death, suggesting involvement of a p53-independent cell death pathway.

Based on flow cytometric analyses khat was shown to induce phosphorylation and acetylation of p53 in both khat-sensitive MOLM-13 cells and the khat-resistant MV-4-11 cell line. These PTMs are reported to mediate stabilization and accumulation of the p53 protein, by inhibiting Mdm2-mediated ubiquitination and degradation (149, 150). However, accumulation of the full length p53 isoform, p53 FL, was only seen in MOLM-13, whereas the levels of the truncated β/γ p53 isoforms were reduced. A similar p53 isoform modulation was previously reported in AML patients receiving induction chemotherapy (192). The expression of various p53 isoforms represents a means of regulating the transcriptional activity of p53 and the cellular response to stress signals (215).

p53 was shown to induce transcription of p21 and Mdm2, whereas levels of pro-apoptotic Bcl-2 family proteins like Bax and Noxa remained constant. In addition to inducing transcription of pro-apoptotic genes, p53 is known to have direct death promoting functions, and p53 and Mcl-1 have been shown to have opposite effects on mitochondrial OM stability (163). The results in paper I and II could therefore suggest that khat destabilized the mitochondria through elevated p53 and down-regulation of Mcl-1 in MOLM-13. However, pre-incubation with pifithrin μ, which inhibits the transcription-independent activity of p53 in mitochondria (216), did not affect the level of khat-induced cell death in MOLM-13 (results not shown). This further suggested that khat-mediated cell death was independent of p53 in AML cell lines. In paper II, experiments using p53 -/- bone marrow

cells and MOLM-13 p53 knock-down cells demonstrated that p53 was not necessary in khat-mediated cell death. Further, protein analyses of p63 and p73 indicated that these p53 family members remained unaltered (217, 218).

The p53 protein was only induced by the death-inducing khat concentration in MOLM-13, and not by sub-lethal dilutions or in the khat-resistant MV-4-11 cell line. In paper III, the cytotoxic khat concentration induced p53 in a subset of normal peripheral blood leukocytes and was shown to induce cell death and reduced proliferation of PBMCs. Together these observations suggested that p53 could represent an indicator for sensitivity to khat and its cytotoxic constituents. We therefore suggested that the p53 isoform pattern and its post-translational modifications could be useful in evaluation of biological effects of extracts, and when screening for novel chemical probes and experimental therapeutics.

5.5 Cytotoxic khat constituents

Alkaloids have contributed the largest number of substances to the modern pharmacopeia, and the vinca alkaloids represent one of the main classes of compounds used as anti-cancer therapeutics (1). The khat alkaloid cathinone was termed a natural amphetamine due to its similarities with amphetamine, and various amphetamines have been shown to induce apoptosis in neuronal cells (219, 220). The alkaloid fraction in khat was indicated to mediate cytotoxic effects in an early study (25), and cathinone was shown to have a mitodepressive effect on dividing cells in root tips, indicating a cytotoxic potential (221). These observations suggested that the khat amphetamines could account for khat-mediated cell death in AML cell lines (63). In agreement with this, we previously observed that the khat amphetamines were cytotoxic to HL-60 cells, and suggested that these compounds were partly responsible for the cell death inducing potential of khat (62). In contrast, the khat amphetamines did not induce cell death in primary oral fibroblasts and keratinocytes (59). However, the results in paper IV in this study showed limited cytotoxic effects by the khat amphetamines in HL-60 and MOLM-13 cells, and demonstrated that other compounds were responsible for the cytotoxicity of the khat extract.

There are several possible explanations for the contradiction between our previous results and the findings in paper IV. The HL-60 cell line used in the previous study is no longer available in our laboratory, and it is possible that the new cell clone used in this thesis is slightly different. Cultivation of cell lines is known to represent a selection process, which

ultimately could result in an altered genetic expression profile of the cells. In addition, the previous study was performed in a different laboratory, suggesting that the experiments could have been performed under slightly different conditions (CO_2 levels, batch of FBS etc.) that might have affected the results.

When comparing the cytotoxicities of the khat extracts from 2005 and 2007 in AML cell lines, the extract from 2005 was observed to be most toxic (results not shown). The concentrations of khat amphetamines in the 2005 extract were lower than their concentrations in the less toxic extract from 2007 (Table 5). These observations further suggested that the cytotoxic potential of khat was not due to the content of khat amphetamines, in agreement with the findings in paper IV. However, we observed an increased signal with the WST-1 viability/proliferation assay in cathinone-exposed MOLM-13 cells in paper IV. This could indicate increased levels of cellular ROS, and hence a cytotoxic effect, or reflect onset of cell differentiation (Herfindal, unpublished data).

When evaluating early intracellular responses and cytotoxic potentials of khat and the khat amphetamines in normal peripheral blood cells in paper III, only khat was observed to induce stress proteins, and shown to induce cell death and reduced proliferation. Cancer cells are known to be more sensitive to cell death-induction compared to normal cells when exposed to drugs targeting deregulated pathways (222). Since the khat amphetamines were observed to be relatively non-toxic to AML cell lines in this study, it was not surprising that the normal blood cells appeared resistant. On the contrary, the compounds were shown to stimulate cell division, with norephedrine mediating significant increased proliferation of PBMCs. This observation agreed with a previous report where cathinone was demonstrated to stimulate B-cells (41).

In paper IV, fractionation of the khat extract and bio-guided screening for cytotoxicity in HL-60 and MOLM-13 cells, led to the identification of a phenylpropanoid glycoside indicated to represent the major cytotoxic constituent in the extract. The molecule was suggested to consist of two central phenylpropanoid glycosides with five attached comaryl-groups. Phenylpropanoid glycosides are common in plants, and known to possess biological activities including anti-viral, anti-microbial and anti-cancer effects. Coumarin derivatives have been reported to inhibit proliferation of leukemic cells and to induce apoptosis (223). Zimmermann and Zneden reported of a phenylpropanoid glycoside with four p-coumarol esters linked to the sugar moiety, shown to possess anti-cancer activity (224, 225). More than 100 phenylpropanoid glycosides have been identified and characterized, but most plants

contain limited quantities of these potential therapeutics (226). However, further studies need to be done in order to fully identify the molecular structure of the cytotoxic khat component(s) described in paper IV.

5.6 Concluding remarks

This study has elucidated mechanisms underlying khat cytotoxicity in AML cell lines, and identified possible cellular and molecular targets with potential within anti-cancer treatments. When compared with the anti-cancer therapeutics CPT, khat was shown to mediate a different and specific cell death phenotype. However, the exact sequence of cellular and molecular events mediating cell death is still not determined. A complex cytotoxic plant extract is likely to activate several cellular pathways simultaneously, which will make it difficult to determine the pathway of cell death-execution.

Analyses of p53 protein protein isoforms and its PTMs were suggested to represent a means for characterizing cytotoxicity by khat and other complex extracts. In contrast to khat, the natural khat amphetamines were not observed to be significantly cytotoxic to AML cell lines or to PBMCs, and were shown to stimultate proliferation of the latter. Bio-guided screening of khat fractions in AML cell lines led to the identification and partial characterization of the main cytotoxic component(s) in the khat extract, which was suggested to be a phenylpropanoid glycoside.

6. FUTURE PERSPECTIVES

The cellular and molecular mechanisms underlying khat-mediated cytotoxicity should be further investigated. The molecular basis for resistance to khat should be determined, in order to elucidate the pathway(s) mediating cell death. Khat was shown to activate components of the receptor-mediated death pathway, and studies should assess the involvement of death receptors.

Khat was shown to trigger autophagy, and its role in cell death-induction should be investigated. Further, the involvement of p38 in the autophagosomal-lysosomal pathway and khat-mediated cell death should be explored. The khat-induced modulation of p53 should be compared with other cytotoxic compounds, in order to evaluate whether specific p53 isoform patterns could indicate the underlying mechanisms.

The effects of khat and the khat amphetamines on intracellular signaling in normal peripheral leukocytes should be further studied. Experiments evaluating the duration of the transient protein modifications should be performed, in order to elucidate activation or attenuation of intracellular signaling cascades. In addition, studies should evaluate whether the khat amphetamines could function as specific inhibitors of signaling pathways.

The exact structure of the main cytotoxic compound(s) in khat should be determined. The cytotoxic effects of this compound should be investigated in leukemic cells, in normal peripheral blood cells, in addition to its effects on normal cells of the oral cavity and gastrointestinal tract. To further evaluate the anti-cancer potential of khat constituents more experiments with primary AML patient samples should be carried out, and the compound(s) could eventually be tested in established animal models of AML.

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