Mechanisms of Betulinic acid-induced cell death

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Mechanisms of Betulinic acid-induced cell death

Lisette Potze

2015

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Voor het bijwonen van de openbare verdediging van het proefschrift:
Mechanisms of Betulinic acid-induced cell death

door
Lisette Potze

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“An expert is someone who has made all the mistakes which can be made, in a very narrow field.”

Niels Bohr

aan mijn ouders
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Chapter 1

Introduction

"Begin at the beginning," the King said gravely, "and go on till you come to the end; then stop."

Lewis Carroll, Alice in Wonderland
Introduction

1. Cancer

Cancer is a group of diseases characterized by unregulated cell growth and the invasion and spread of cells from the site of origin, or primary site, to other sites in the body. Cancer has defects in regulatory circuits that control normal cell proliferation and homeostasis, resulting in uncontrolled expansion of cells. Processes that contribute to the overall net cell number are cell proliferation and elimination of cells by programmed cell death (discussed below). Cancer is a genetic disease, where mutation in oncogenes (genes that have dominant gain of function due to mutation, amplification and/or overexpression) and tumor suppressor genes (genes with recessive loss of function mutations, deletion and/or epigenetic silencing of these genes) dictate the defects in cellular expansion.\(^1\)\(^,\)\(^2\) In 2000, Hanahan and Weinberg proposed that cancer arises through a multistep, mutagenic process in which oncogenes and tumor suppressor genes play an important role, whereby cancer cells acquire essential alterations (hallmarks) in the physiology of the cell that combined result in malignant growth.\(^\text{3}\) These hallmarks include self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis and evasion of apoptosis. Conceptual progress has been made after the description of the first hallmarks and therefore a decade after these descriptions were proposed, Hanahan and Weinberg added two additional characteristics and two emerging hallmarks. These include: deregulated cellular energetics, evasion of anti-cancer immunity, tumor promoting-inflammation and genome instability and mutation.\(^\text{4}\) The hallmarks of cancer have attributed to our understanding of the disease and are a potential target for the treatment of cancer.

In this introduction we will focus on the following hallmarks: evading apoptosis and deregulated cellular energetics. We provide an overview of cell death mechanisms and how these are altered in cancer, followed by an overview of cell metabolism and its alterations occurring in cancer.

2. Programmed cell death

Programmed cell death (PCD) is an evolutionary highly conserved fundamental biological process. PCD removes old needless cells during tissue formation thereby maintaining tissue homeostasis. Homeostasis is needed to keep internal conditions stable and relatively constant. To maintain homeostasis, PCD also removes damaged or abnormal cells. Several forms of PCD exist and the last few years more accurate definitions of these cell death pathways have been postulated by the Nomenclature Committee on Cell Death (NCCD). These definitions are based on molecular characteristics instead of the classical morphological traits and include extrinsic apoptosis, caspase-dependent or caspase-independent intrinsic apoptosis, autophagic cell death, regulated necrosis and mitotic catastrophe.\(^\text{5}\) Apoptosis is a cell autonomous program of damaged or stressed cells
Chapter 1

resulting in ‘organized’ cell death which can be executed by two distinguishable pathways: the extrinsic pathway and the intrinsic pathway.

2.1. Extrinsic apoptosis

The extrinsic pathway, also known as death receptor pathway, is activated by binding of a so-called death ligand to its receptor belonging to the tumor necrosis factor (TNF) superfamily. Members of the TNF family share similar cysteine-rich extracellular domains and have a cytoplasmic domain about 80 amino acids in size called the death domain.\(^6\) The best characterized ligands are FasL/CD95L, TNF-alpha and TNF-related apoptosis inducing ligand (TRAIL). The corresponding receptors are Fas/CD95, TNFR1-2 and TRAILR 1-4, respectively.\(^5,\ 7-9\) Binding of a death receptor ligand to its receptor results in the recruitment of adaptor protein Fas-associated death domain (FADD) and in case of TNFR-1 like proteins, TNFR-associated death domain (TRADD) is first recruited followed by FADD.\(^10\) Besides FADD and TRADD, cellular inhibitor of apoptosis proteins (cIAPs), cellular FADD-like interleukin-1β-converting enzyme (FLICE)-like inhibiting proteins (cFLIPs), receptor-interacting protein kinase 1 (RIPK1 also known as RIP1 ), E3 ubiquitin ligases and procaspase-8 and pro-caspase-10 are recruited to the death domain.\(^5\) The resulting complex, named ‘death-inducing signaling complex (DISC), constitutes a platform that regulates the activation of caspase-8 and caspase-10.\(^5,11,12\) Depending on cell type (type 1 or type 2 cells) two different routes of further downstream pathways are known.\(^13,14\) In type 1 cells, active caspase-8 directly activates caspase-3 and thereby trigger the executioner phase. In type 2 cells, caspase-8 mediates the cleavage of BH3 interacting domain death agonist (BID) this results in the generation of truncated BID, wich translocates to the mitochondria and releases cytochrome c and results in mitochondrial outer membrane permeabilization (MOMP).\(^5\) These pathways are tightly regulated. At the level of DISC inhibitory proteins like cFLIPs and cIAPs can prevent the recruitment of procaspase-8 by competitive binding to the death domain of FADD or by inhibition of caspase-8 activation within the cFLIP-containing DISC.\(^10\) In type 2 signaling, apoptosis can, in addition to regulation at the DISC, be regulated by anti-apoptotic BCL2 proteins like BCL2 or BCL-XL to block the truncated BID-mediated apoptosis. Another route of inhibition is by inhibitor of apoptosis proteins (IAPs), which prevents caspase-9 activation.\(^10\) These inhibitory proteins themselves are counteracted by mitochondrial proteins such as SMAC/DIABLO. More information about mitochondrial involvement in apoptosis and the proteins involved is described below.

2.2. Caspase-dependent and caspase-independent intrinsic apoptosis

Intrinsic apoptosis is triggered by intracellular stress signals such as growth factor withdrawal, DNA damage, oxidative stress or oncogene activation.\(^15\) Intrinsic apoptosis is defined as a cell death process that is mediated by mitochondrial outer membrane permeabilization (MOMP) and is consequently associated with loss of mitochondrial
Introduction

transmembrane potential, release of mitochondrial intermembrane space proteins (i.e. cytochrome c) into the cytosol and loss of mitochondrial respiratory chain activity.\textsuperscript{[15]}

Currently it is still debated how cytochrome c is released from the mitochondria, however it is known that the release is tightly regulated by the B cell CLL/lymphoma-2 (BCL-2) family proteins which consists of pro-apoptotic proteins and pro-survival proteins.\textsuperscript{[15, 16]} The BCL-2 family is a large set of proteins which all contain at least one conserved BCL-2 homology (BH) domain. The anti-apoptotic proteins (BCL-2, BCL-XL, BCL-W, Mcl1 and A1) share four BCL-2 homology domains (BH1–BH4) among each other, with the exception of Mcl-1 that contains only three BH domains.\textsuperscript{[17]} The anti-apoptotic proteins are generally integrated within the outer mitochondrial membrane (OMM) but can also found in the cytosol and endoplasmic reticulum.\textsuperscript{[18]}

The pro-apoptotic BCL2 family members can be divided into two groups: BAX like molecules (BAX, BAK, BOK, BCL-G and BFK, also known as effector proteins) which contains multiple (at least two) BH domains (BH1, BH2, BH3 and BH4).\textsuperscript{[16, 18]} The second group of proteins are the BH3-only proteins (amongst others: BID, BIM, BAD, PUMA (p53 upregulated modulator of apoptosis), Noxa, BIK, HRK and BMF) which share only the BH3 domain.\textsuperscript{[17]}

In initiation of intrinsic apoptosis, BAK/BAX activation is crucial. Oligomerization of BAK/BAX results in proteolipid pores within the OMM and thereby promote MOMP with subsequent cytochrome c release. Combined deletion of BAK/BAX prevents release of cytochrome c and results in resistance to all death stimuli of the intrinsic apoptosis pathway.\textsuperscript{[19]} BH3 only proteins are cell death initiators, which are activated by transcriptional or posttranslational mechanisms in a tissue-restricted and signal specific manner.\textsuperscript{[20-22]} They act upstream of BAK/BAX and can be subdivided based on their ability to interact with the anti-apoptotic proteins or both anti-apoptotic as well as BAK like molecules (the effectors). BH3-only proteins that only bind and inhibit the anti-apoptotic proteins are called “sensitizers” or “de-repressors” (BAD, BIK, Noxa and HRK), while BIM, BID and PUMA, known as “activators”, can bind the anti-apoptotic proteins as well as directly bind to BAK/BAX and inducing oligomerization and MOMP.\textsuperscript{[18, 23]} Each sensitization/de-repressor protein has a unique binding profile for the anti-apoptotic proteins. Sensitization lowers the threshold for the activation of BAK/BAX and MOMP, but does not cause apoptosis itself. In de-repression, a direct activator is bound by an anti-apoptotic protein, and a subsequent BH3-only protein releases the activator to promote MOMP.\textsuperscript{[18]}

Upon MOMP, the released cytochrome c binds apoptotic protease activating factor 1 (APAF-1), inducing its conformational change and oligomerization leading to the formation of a caspase activation platform called the apoptosome. The apoptosome recruits, dimerizes and activates caspase 9 (an initiator caspase), which can cleave and activate caspase 3 and caspase 7 and results in apoptosis.\textsuperscript{[24]} MOMP results also in the release of mitochondrial release of second mitochondria-derived activator of caspase (SMAC, also known as DIABLO)
and OMI (also known as HTRA2) which block X-linked inhibitor of apoptosis protein (XIAP)-mediated inhibition of caspase activity.\(^{(24-27)}\)

**Tumor suppressor protein p53**

Upon cell stress and DNA damage, a tumor suppressor gene called p53 is activated, and has several downstream effects including cell cycle arrest, DNA repair, inhibition of angiogenesis and apoptosis. With these effects the cells get a chance to repair the damage. p53 induces the expression of genes that encode for death receptors and pro-apoptotic proteins (i.e. Fas receptor, BAK and BAX)\(^{(28-31)}\) while on the other hand it represses the expression of anti-apoptotic proteins (i.e. BCL-2, BCL-XL and IAPs) The pro-apoptotic protein PUMA is a direct target of p53.\(^{(32)}\) It has been proposed that p53 can also induce apoptosis independently of its transcriptional activity by activating BAX in the cytoplasm and subsequent cytochrome c release.\(^{(33)}\) Taken together one can conclude that p53 is a key regulator in apoptosis signaling.

**Figure 1: Apoptotic signaling.** Induction of apoptosis by the extrinsic pathway depends on the binding of ligand to its receptor. A death-inducing signaling complex is formed and active caspase 8 generated, which can be inhibited by high levels of c-FLIP. Crosstalk between extrinsic and intrinsic pathway can occur through BID cleavage, which leads to activation of the pro-apoptotic BCL2 family proteins BAX and BAK, resulting in MOMP. The intrinsic apoptosis pathway depends on MOMP and apoptosome formation which results in caspase 3 activation and apoptosis. Intrinsic apoptosis is tightly regulated by the balance between the anti-apoptotic and pro-apoptotic members of the BCL-2 family. Abbreviations: APAF1, apoptotic protease-activating factor 1; DISC, death-inducing signaling complex; FADD, Fas-associated protein with death domain; c-FLIP, cellular FLICE-like inhibitory protein; FasL, Fas ligand; MOMP, mitochondrial outer membrane permeabilization; SMAC, second mitochondria-derived activator of caspase; XIAP, X-linked inhibitor of apoptosis protein.
2.3. Autophagy

Autophagy is a highly conserved cellular proteolytic (lysosomal) degradation process in which cytosolic components are first encapsulated by a membrane and subsequently degraded in the lysosome, thereby providing new building blocks for the cell and is typically induced upon nutrient starvation. In cells without stress, basal levels of autophagy take place to perform homeostatic functions such as protein and organelle turnover.

There are three forms of autophagy which are mediated by distinct mechanisms. In chaperone-mediated autophagy, targeted proteins are translocated across the lysosomal membrane in a complex with chaperone proteins (i.e. Hsc-70) that are recognized by the lysosomal membrane receptor lysosomal-associated membrane protein 2A (LAMP-2A), resulting in their unfolding and degradation.\(^{34, 35}\) In contrast, micro- and macroautophagy involves the sequestration of cargo (both selective and non-selective mechanisms) via a sequestering membrane. In microautophagy the sequestration of cytosolic components occurs directly by the lysosomes, while in macroautophagy the cargo is sequestered within a unique double membrane cytosolic vesicle (an autophagosome) which later on fuses with a lysosome for degradation of the cargo.\(^{36, 37}\) Selective degradation involves targeting specific cargoes (i.e. organelles (i.e. damaged mitochondria) or invasive microbes).

Macro autophagy starts with an isolated membrane (phagophore), which is suggested to be derived from lipid bilayer contributed by endoplasmic reticulum, trans-Golgi system and endosomes.\(^{38}\) The phagophore expands so it can engulf cytosolic cargo (i.e organelles, ribosomes, protein aggregates) to form a double-membraned autophagosome.\(^{39}\) The autophagosome fuses with a lysosome, promoting degradation of the cargo by lysosomal proteases. The resulting amino acids and other by-products of degradation are released to the cytosol via lysosomal permeases and lysosomal transporters where they can be used as building blocks.\(^{39}\) Canonical autophagy involves five steps where autophagy related proteins play an important role: (1) initiation (which depends on ULK1 complex (ULK1, ATG13 and ATG17) and receives stress signals from mTOR complex 1), (2) nucleation (which depends on the BECLIN1–PtdIns3KC3–ATG14L complex), (3) elongation and closure of autophagosome (which depend on ATG12–ATG5 (including ATG7) and LC3–PE conjugation systems), (4) recycling (which depends on ATG9) and (5) degradation by fusion to the lysosome.\(^{40}\) The formation of functional autophagosomes can bypass some of these steps, although these alternative mechanisms are currently under debate and are named non-canonical autophagy. There are some forms of non-canonical autophagy pathways that have been identified under certain cellular circumstances: one that bypasses the proteins involved in elongation and closure (ATG7, ATG5 and LC3) and others that bypass proteins that are important for initiation (ULK1) and nucleation (BECLIN1). (reviewed in \(^{40}\))

The capacity for large scale degradation carries a certain risk for cells, as unregulated degradation of cytoplasm or organelles is likely to be lethal.\(^{37}\) Therefore induction of
autophagy is tightly regulated by several proteins. Two evolutionarily conserved nutrient sensors play a role in autophagy regulation: (1) the mammalian target of rapamycin (mTOR) kinase which under normal conditions inhibits autophagy). When mTORC1 kinase activity is inhibited autophagosome formation occurs.\(^{(41, 42)}\) Second the eukaryotic initiation factor 2α (eIF2α) kinase Gcn2 and its downstream target Gcn4 (a transcriptional transactivator of autophagy genes) turn on autophagy during nutrient depletion.\(^{(43)}\) Also p53 plays a role in regulating autophagy. As a transcription factor in the nucleus it can activate proteins (i.e DRAM) which activates autophagy.\(^{(44)}\)

![Diagram of autophagy forms](image)

Figure 2: An overview of several forms of autophagy. Macroautophagy, microautophagy and chaperone mediated autophagy are depicted. See text for detailed information.

### 2.4. Autophagic cell death

The definition of autophagic cell death (according to NCCD) is cell death that is mediated by autophagy and as such can be suppressed by the inhibition of the autophagic pathway by genetic means (gene knockout or RNA interference for i.e. ATG5, ATG12) and/or chemicals (i.e., agents that target VPS34).\(^{(5)}\) Certain dying cells display the morphological hallmarks of autophagy, however the question whether autophagy has a causative role in cell death has been debated. It has been suggested that under specific circumstances that depend on the nature of the stimulus, the duration and its amplitude, extensive autophagy may cause cell death.\(^{(45)}\) The discovery of chemical compounds (i.e 3- methyladenine (3-MA) and wortmannin) that can inhibit autophagy resulted in several reports about autophagic cell death. These reports describe the existence of a caspase-independent cell death which
proceeded with an accumulation of autophagosomes and increased lysosomal activity, and using the inhibitors of autophagy a reduction in cell death was observed. For example, a study using oncogenic Ras-induced death of glioma or gastric cells showed cell death in the absence of caspase activation, which was not inhibited by the overexpression of anti-apoptotic BCL-2 protein.\textsuperscript{(46)} Another study showed reduced cell death using 3-MA for anti-estrogen induced cell death in MCF-7 cells.\textsuperscript{(47)} However, in these studies, autophagy occurred in cells thought to die by apoptosis, and it was presumed that autophagy triggered apoptosis, instead of playing a causative role. Moreover, 3-MA is not specific for class III PI3K kinases and can inhibit other kinases as well as inhibiting the permeability transition in mitochondria.\textsuperscript{(48)} Thus, it is not possible to directly implicate autophagy in death execution from these 3-MA inhibitor studies. A different approach was taken with RNA interference (RNAi) of two ATG genes, ATG7 and BECLIN1, this resulted in cell death in mouse L929 cells and in macrophages when caspase inhibitor zVAD was used.\textsuperscript{(49, 50)} RNAi against ATG5 and BECLIN1 resulted in an inhibition in cell death in BAK/BAX knock out murine embryonic fibroblasts (MEFs) treated with etopside or staurosporin.\textsuperscript{(51)} Notably, in these studies, apoptosis was blocked and the ATG gene RNAi blocked the cell death. These findings exclude that autophagy is triggering apoptosis, nonetheless they raise the question if autophagy could be a cell death mechanism is cells whose apoptotic machinery is intact.\textsuperscript{(52)} In etopside-treated wild type MEFs (which die via apoptosis) only small amounts of autophagy were detected and inhibition of autophagy by 3-MA did not reduce the etopside-induced cell death. This indicates that autophagy cannot perform cell death induction unless apoptosis is blocked.\textsuperscript{(51)} It could be that cells prefer to die via apoptosis but will die by an alternative route if the stimuli is harsh enough.\textsuperscript{(53)} A different possibility is that apoptotic cell death is faster than autophagic cell death and therefore the last one is observed only in apoptotic deficient cells.\textsuperscript{(52)} So up to now it is still debatable if autophagic cell death occurs in cells which have intact apoptosis.

\subsection*{2.5. Regulated necrosis}

Necrosis is a form of passive cell death that is induced upon strong insults such as mechanical injury of cells. Typical features include swelling, rupture of organelle membranes as well as the outer cell membrane and as a result the cell contents are released, often causing inflammation \textit{in vivo}.\textsuperscript{(54)} In the past, necrosis has been considered as an accidental cell death mechanism defined by the absence of morphological characteristics of apoptosis and autophagy.\textsuperscript{(55)} However, several laboratories have now shown that necrosis can occur in a regulated manner.\textsuperscript{(56)} Under selected circumstances regulated necrosis can be induced by several triggers like excitotoxins, ligation of death receptors and alkylating DNA damage.\textsuperscript{(57-60)} Regulated necrosis was first described that it include caspase inhibition (especially caspase-8) by pharmacological agents (i.e., chemical caspase inhibitors like Z-VAD-fmk) or genetic manipulations (i.e., RNA interference or gene knockout), followed by a block in degradation of receptor interacting protein 1 (RIP1) and
RIP3. The latter two proteins in turn form a multiprotein complex called the nescrosome, which further activates players for execution of necrotic cell death.\textsuperscript{56} However later research showed that regulated necrosis does not occur only in caspase-incompetent cells upon the activation of the receptor interacting protein 1 (RIPK1) homolog RIPK3. There are multiple molecular circuits that can drive regulated necrosis including (but not limited to) necroptosis (RIP1 dependent, inhibitible by necrostatin 1 form of TNFR1 induced regulated necrosis), mitochondrial permeability transition (MPT)-dependent regulated necrosis (relies on cyclophylin D, reviewed in \textsuperscript{62}), and parthanatos (a non-apoptotic cell death subroutine that critically relies on the (hyper)activation of poly(ADP-ribose) (PAR) polymerase 1 (PARP1)).\textsuperscript{62, 63} Over the past years it has become clear that regulated necrosis plays a role in physiological scenarios (e.g., embryonic development) and pathological settings (e.g., ischaemic injury, neurodegeneration and viral infection). This led to the suggestion that patients might benefit from pharmacological modulation of regulated necrosis.\textsuperscript{11, 56, 64}

2.6. Lysosomal cell death

Lysosomes are the main compartment for intracellular degradation and subsequent recycling of cellular constituents. The degradation is carried out by a number of acid hydrolases (i.e. phosphatases, nucleases, glycosidases, proteases, peptidases, sulfatases and lipases) capable of digesting all major cellular macromolecules.\textsuperscript{65, 66} The best-studied lysosomal hydrolases are the cathepsin proteases. Based on their active site amino acid, i.e. cysteine (B, C, H), aspartate (D and E) and serine (G) cathepsins they are divided into three subgroups.\textsuperscript{67} Lysosome membrane permeabilization and the consequent leaking of hydrolases (mainly cathepsin proteases) into the cytosol, can initiate the intrinsic apoptosis pathway\textsuperscript{68} but also can trigger caspase-independent non-apoptotic cell death pathway, indicating that the lysosomal hydrolases can act as initiators as well as effectors of PCD.\textsuperscript{66, 69, 70} This form of cell death where lysosomal hydrolases are the key activation step is called lysosomal cell death.\textsuperscript{66, 71} Although the induction of lysosomal cell death is clearly distinct from necrosis and apoptosis, the execution phase is similar.

The function of lysosomal cathepsin proteases is not limited to intralysosomal protein turnover and degradation of extracellular matrix when secreted. The last years many specific functions of the cathepsin proteases have been discovered. These specific functions include roles in cell death, bone remodeling, antigen presentation, epidermal homeostasis, angiogenesis and cancer cell invasion.\textsuperscript{66, 72-75} In cancer, cysteine cathepsins (i.e. B, C, H) are upregulated by a variety of mechanisms like gene amplification, transcription regulation, post-transcriptional modifications and epigenetic regulation.\textsuperscript{76} Trafficking and subcellular localization of these cysteine cathepsins changes during neoplastic progression, resulting in active and inactive forms which have pro-oncogenic effects. The enhanced secretion of these cathepsins initiate tumor growth, migration, invasion, angiogenesis and metastasis that increase neoplastic progression. This role of cathepsins in cancer has been well studied \textit{in vitro and in vivo}. (reviewed in\textsuperscript{76}) At
the same time the upregulation of these cathepsins may sensitize cells towards the lysosomal cell death pathway. Especially cell death could occur in tumor cells with several defects in the classical apoptosis pathways.\(^{(77)}\)

Cancer cells have developed several strategies to protect them from lysosomal membrane permeabilization and the acid hydrolases leaking into the cytosol. One strategy is upregulation of lysosomal protease inhibitors, the best characterized inhibitors are the squamous cell carcinoma antigens (SCCA) 1 and 2. SCCA 1 and 2 are tumor-associated proteins which can inhibit serine proteases and whose levels are used in diagnosis of squamous cell carcinoma.\(^{(78)}\) Cysteine cathepsins can be inhibited by cystatin A and B and serine protease inhibitor 2A.\(^{(66, 79, 80)}\) In cystatin B-deficient mice an increase in apoptosis was observed, demonstrating the importance of these inhibitors in preventing cell death.\(^{(81)}\)

A second strategy is upregulation of heat shock protein 70 (Hsp70), heat shock proteins are molecular chaperones that interact with several proteins to assist in their folding, stability and function. Hsp70 stabilizes lysosomes by binding to phospholipid bis-(monoacylglycerol)-phosphate (BMP), which is highly present in intraluminal membranes of the lysosomes.\(^{(70, 82, 83)}\) BMP is a well described docking lipid for enzymes and cofactors involved in lysosomal degradation of sphingolipids.\(^{(82)}\) One of these enzymes is acid sphingomyelinase (ASM), which activity depends on its recruitment to the intralysosomal membranes by BMP \(^{(84)}\), and plays a crucial role in the Hsp70-mediated stabilization of lysosomes.\(^{(83)}\) Hsp70 mediates an increase in ASM activity, which leads to higher lysosomal ceramide content and increased lysosomal stability.\(^{(70)}\) How Hsp70 affects the stability of the lysosome is still unclear but the hypothesis is that the change in lipid composition of the intralysosomal membranes has an influence on the stability of the entire lysosome. It could be that an ASM dependent increase of ceramide alters the properties of membranes in such a way that it has a positive effect on the stability of the outer membrane.\(^{(70)}\)

In light of the dependence on these survival strategies further elucidation of these mechanisms controlling lysosomal membrane stability could lead to potential cancer drug targets.

3. Cell death in cancer

3.1. Apoptosis and cell death

The transforming effects of proto-oncogenes (i.e. Myc) that mediate unrestrained cell proliferation are countered by “intrinsic tumor suppressor mechanisms” that most often trigger apoptosis.\(^{(85)}\) Defects in the apoptotic machinery play an important role in oncogenesis, allowing neoplastic cells to survive over intended lifespans, subverting the need for exogenous survival factors and providing protection from oxidative stress and hypoxia as the tumor mass expands.\(^{(86)}\) Apoptosis defects are considered an important complement of pro-oncogene activation, as many deregulated oncoproteins (i.e. Myc, Cyclin-D1) that drive cell division also trigger apoptosis.\(^{(87)}\) For example, Myc constantly
induces cell proliferation and apoptosis. To facilitate myc-driven tumorigenesis, inhibition of apoptosis (e.g., loss of function of p53 or overexpression of BCL2) is required.\textsuperscript{(85)} Mutations that affect the extrinsic pathway of apoptosis are for example mutations in death receptor genes. Epigenetic silencing of death receptors occur like epigenetic silencing of TRAIL-R1 results in apoptosis resistance in gliomas\textsuperscript{(88)} and ovarian cancer.\textsuperscript{(89)} Furthermore, cFLIP is overexpressed in many tumors and thereby block caspase 8.\textsuperscript{(90, 91)} Beside blocking of caspase-8, loss of caspase-8 expression (i.e. by epigenetic silencing or loss of heterozygosity (LOH)) has been observed.\textsuperscript{(92)} Downregulation/loss of Fas expression has been also found in a variety of tumors including melanoma, lung adenocarcinomas and esophageal cancer.\textsuperscript{(93-95)} A well-known alteration in the resistance to intrinsic apoptosis are mutations in the tumor suppressor gene p53. This results in loss of induction of BAX and PUMA and to some extent NOXA and as a consequence a block in apoptosis.\textsuperscript{(96)} Next to loss of p53-dependent apoptosis, overexpression of anti-apoptotic BCL-2 or BCL-XL probably occurs in more than half of all cancers\textsuperscript{(97)} rendering tumor cells resistant to apoptotic stimuli, including most cytotoxic anticancer drugs. Cancer cells can acquire resistance to apoptosis by down regulation or mutation of pro-apoptotic proteins such as BAK and BAX. For example, haploid loss of BAX results in accelerated tumor progression in breast cancer mice models\textsuperscript{(98)} and in myc-mediated lymphogenesis.\textsuperscript{(99)} In tumors with microsatellite instability, several mutation in BAX are observed leading to decreased apoptosis and thus increased tumor progression.\textsuperscript{(100, 101)} In general, cancer cells have acquired many different mutations that result in a diminished or complete block in apoptosis, thereby promoting tumor progression.

\section*{3.2. Autophagy and cancer}

The regulation of autophagy overlaps closely with signaling pathways that regulate tumorigenesis. Tumor suppressor genes like p53, PTEN, TSC1 and TSC2 involved in the upstream inhibition of mTOR signaling, can stimulate autophagy. In contrast oncogene products like class I PI3K, AKT and BCL-2 can activate mTOR signaling and inhibit autophagy.\textsuperscript{(102, 103)} In cancer, anti-apoptotic proteins BCL-2 and BCL-XL are upregulated in many tumors and they inhibit autophagy by binding to the BECLIN-1 autophagy protein.\textsuperscript{(104, 105)} In 40-75\% of the cases of human ovarian, prostate and breast cancer one allele of BECLIN-1 is deleted. This is important in oncogenesis because mice with heterozygous disruption of BECLIN-1 have a diminished autophagy and were more prone to develop tumors.\textsuperscript{(106, 107)} These data suggest that autophagy acts as a tumor suppressive mechanisms, however the mechanism by which autophagy functions in tumor suppression is still unclear. Autophagy also plays a role genomic stability, mono-allelic or bi-allelic loss of BECLIN-1 or ATG5 results in increased DNA damage, gene amplification and aneuploidy in parallel with increased oncogenesis.\textsuperscript{(108)} For the mechanism behind this more research is needed.
3.3. Necrosis and cancer

In (regulated) necrosis cells release many pro-inflammatory signals to the surrounding, resulting in recruitment of immune inflammatory cells\(^{109}\), which have been shown to actively promote tumorigenesis by fostering angiogenesis and cancer cell proliferation and invasiveness.\(^4\) On top of that, necrotic cells release bioactive regulatory factors (i.e IL-1\(\alpha\)) which can directly stimulate the microenvironment to proliferate facilitating neoplastic progression.\(^{110}\)

Thus in general, cancer cells can make evasive adaptations in all different cell death mechanisms or use them to their benefit. These alterations render the cells to resistance to conventional therapies like chemotherapy and radiotherapy.

4. Metabolism

Activation of oncogenes and the loss of tumor suppressor genes promote metabolic reprogramming in cancer. This hallmark of cancer cells is mirrored in enhanced nutrient uptake to supply energy and building blocks for biosynthetic pathways. Moreover, cancer cells are typified by their ability to exhibit metabolic flexibility to sustain their growth and survival when nutrients are limited.\(^{111}\) Over the last decade, researchers started to unravel the precise mechanisms behind the multitude of metabolic changes observed in cancer cells. This has been and still is a formidable task as cellular metabolism in eukaryotes is a complex system with more than 5000 metabolic reactions catalyzed by more than 10,000 enzymes.\(^{112}\) and www.genome.jp/kegg) Metabolism pathways are divided into two main categories, which are energy metabolism pathways (such as glycolysis, Krebs cycle, fatty acid oxidation and oxidative phosphorylation) and biosynthesis pathways (such as lipid and fatty acid synthesis and biosynthesis of nucleotides via the pentose phosphate pathway).

4.1. Energy metabolism

Adenosine triphosphate (ATP) is an energy carrier in all mammalian cells. Cells produce ATP via multiple pathways. Exogenous glucose is via glycolysis converted to pyruvate, resulting in a net gain of two ATP molecules. In the absence of oxygen, pyruvate is further metabolized to lactate without a further gain in ATP molecules. In the presence of oxygen, pyruvate is converted into acetyl CoA and this product can enter the citric acid cycle (also known as Krebs cycle), which is a continuous cycle where acetyl CoA and oxaloacetate are processed by 8 different enzymes. The first conversion is to citrate followed by processing to cis-Aconitate, isocitrate, which is further oxidized to oxalosuccinate and converted to \(\alpha\)-ketoglutarate. \(\alpha\)-ketoglutarate is processed to succinyl-CoA, then to succinate. Succinate dehydrogenase converts succinate into fumarate, which is then processed into \(l\)-malate. The final step involves the oxidation of \(l\)-malate into the starting product oxaloacetate, which can then with a new molecule acetyl CoA restart the cycle. The crucial product of the
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Krebs cycle is NADH and FADH$_2$ which are used to generate ATP via oxidative phosphorylation. Via glycolysis and the Krebs cycle the breakdown of one glucose molecule results in a total of 36 ATP molecules.

Next to glucose other biomolecules can enter the Krebs cycle and tumor cells utilize another source for energy production via the so called glutaminolysis pathway. This pathway shunts glutamine via glutamate into the Krebs cycle at the level of α-ketoglutarate and can therefore also generate NADH and FADH$_2$.

4.2. Biosynthesis

Dividing cells need nucleotides, lipids and amino acids to create new DNA, membranes and proteins. The pentose phosphate pathway (PPP) produces ribose-5-phosphate, which can be used to create nucleic acids and nucleotides. Fatty acids from exogenous sources can be taken up by cells via fatty acids transporters$^{[113]}$ or are synthesized de novo, which under physiological conditions occurs mainly in the liver and to a lesser extent in adipose tissue and in lactating breast.$^{[114]}$ Acetyl-CoA gets carboxylated by acetyl-CoA carboxylase (ACC) to form malonyl-CoA. The malonyl-CoA is further converted by fatty acid synthase (FASN) to saturated long chain fatty acids.$^{[115]}$ The maximum length of de novo synthesized fatty acids via this pathway contains 16 carbon atoms (palmitate C16:0). These fatty acids can undergo modifications to be elongated into longer fatty acids by fatty acid elongases or can be desaturated by stearoyl CoA desaturase 1 (SCD-1) to create unsaturated fatty acids. Unsaturated fatty acids are essential components of cell membranes and desaturation of fatty acids occurs to maintain a healthy pool of fatty acids. These processes are regulated by sterol regulatory element binding proteins (SREBPs), a family of transcription factors that regulate lipid homeostasis by controlling the expression of a range of enzymes (ATP-citrate lyase (ACL), ACC and FASN) required for endogenous cholesterol, fatty acid (FA), triacylglycerol and phospholipid synthesis.$^{[116]}$ SREBPs are regulated by SREBP cleavage activating protein (Scap) and ER-resident protein Insig. In a sterol rich environment, Scap binds to cholesterol in the ER membrane and to Insig. This retains the SREBP-Scap complex in the ER. In sterol low conditions, Scap no longer binds Insig and the SREBP-Scap complex is sorted into COPII-coated vesicles to the Golgi, where SREBP is proteolytic cleaved by site1 (S1P) and site 2 (S2P) proteases which results in the release of the N-terminal transcription factor.$^{[117]}$

Fatty acids can also be a source of energy, when fatty acids are degraded via β-oxidation to generate acetyl-CoA. When acetyl-CoA enters the Krebs cycle energy is produced as described above.
Introduction

Figure 3: Overview of cell metabolism. The main metabolic pathways that contribute to the production of macromolecules in mammalian cells are nucleotide synthesis, the pentose phosphate pathway, glutaminolysis, cholesterol synthesis, fatty-acid synthesis and desaturation and elongation. The enzymes involved in these pathways are shown in bold. See text for more detail. Abbreviations: ACC, acetyl-CoA carboxylase; ACL, ATP citrate lyase; ACO, aconitase CoA; coenzyme A; CS, citrate synthase; ELOVL6, fatty acid elongase 6; FASN, fatty-acid synthase; F1,6BP, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; FH, fumarate hydratase; GLS, glutaminase; G6P, glucose-6-phosphate; G6PD, G6P dehydrogenase; HK, hexokinase; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; IDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; PDK1, pyruvate dehydrogenase kinase; PFK, phosphofructokinase; PKM2, pyruvate kinase M2; PL, phospholipids; SCD-1, stearoyl-CoA desaturase 1; SDH, succinate dehydrogenase TAG, triacylglycerides.

4.3. Energy metabolism and cancer

Over time, more evidence appeared showing the importance of a shift in cellular metabolism in cancer cells. The first discovery of a necessary metabolic alteration has been made > 80 years ago by Otto Warburg. He observed that glycolysis is upregulated in tumor cells even if enough oxygen in the surrounding of the cells is available to create ATP. This phenomenon was coined the Warburg effect.\textsuperscript{118, 119} This very fast but inefficient way to produce energy might constitutes an advantage for tumor growth. Firstly, cells that rely on oxidative phosphorylation need a constant flux of oxygen. In tumors hypoxic circumstances with fluctuating oxygen levels are omnipresent. By upregulating glycolysis tumor cells are not harmed by these oxygen fluctuations.\textsuperscript{120-122} Secondly, the Warburg effect leads to the
production of a high level of lactate acids. These acids are suggested to condition the microenvironment of the tumor, favor tumor invasion and suppress anti-cancer immune effectors.\(^{120,123-125}\) Third, cancer cells use intermediates of the glycolytic pathway i.e. ribose sugars for nucleotides, glycerol and citrate for lipids, nonessential amino acids and via the pentose phosphate pathway NADPH.\(^{120,126,127}\)

In glycolysis, hexokinases catalyzes the essentially irreversible first step in this pathway where glucose is rapidly phosphorylated to glucose-6-phosphate. Hexokinases are strongly upregulated in cancer.\(^{128}\) Hexokinase (HK)-II is located in the mitochondria and bound to the outer membrane via the voltage-dependent anion channel (VDAC). HK-II binds ATP (brought to HK-II by VDAC) and the glucose to produce glucose-6-phosphate. Tumor cells have multiple genetic, epigenetic, transcriptional and post-translational strategies to enhance expression and function of hexokinase (HK) II. (reviewed in \(^{128}\)) Besides playing a role in glycolysis HK-II via its mitochondrial location also suppresses death of cancer cells. Binding of HK-II to VDAC diminish the availability of free VDAC sites that can interact with activated pro-apoptotic molecules.\(^{129,130}\)

Glucose provides the cell with acetyl CoA, which can be converted to citrate which is shuttled out of the mitochondria for \textit{de novo} fatty acid synthesis, however continuous export of citrate introduces a deficiency to the Krebs cycle which must be replaced by an anaplerotic flux to maintain fatty acid synthesis to take place.\(^{131}\) In glutaminolysis, glutamine is shuttled into the Krebs cycle where it is converted by glutaminases to glutamate. This route is typically bidirectional (glutamine synthetase to create glutamate) but in cancer glutaminases are overexpressed and/or glutamine synthetase are suppressed, favoring the reaction to create glutamate.\(^{132-134}\) Glutamate is converted into \(\alpha\)-ketoglutarate and thereby replenishing the intermediates in the Krebs cycle. The citrate produced can be cleaved to generate oxaloacetate which is converted into malate and ultimately lactate. Glutamine can also be converted into lactate when mitochondrial malate is exported to the cytoplasm. In tumor cells this pathway is a major source of NADPH.\(^{131}\)

Under normal conditions the transcription factor hypoxia-inducible factor 1\(\alpha\) HIF-1\(\alpha\) (active subunit of HIF-1) is posttranslational modified by prolyl hydroxylation, which promotes association with the von Hippel-Lindau (VHL) tumor suppressor resulting in degradation of HIF-1\(\alpha\) by ubiquitination.\(^{126}\) During hypoxia a process involving reactive oxygen species (ROS) generated in the mitochondria, inhibits the prolyl hydroxylation resulting in stabilization of HIF-1\(\alpha\)\(^{135}\) which stimulates cells to consume glucose and produce lactate.\(^{136}\) Stabilization of HIF-1\(\alpha\) during normoxia occurs in tumors by mutations in VHL, or mutations in succinate dehydrogenase and fumarate hydratase (Krebs cycle intermediate enzymes) Active HIF-1\(\alpha\) can enter the nucleus and activates transcription of glucose transporters, glycolytic enzymes (i.e. pyruvate dehydrogenase kinase 1 (PDK1)) and lactate dehydrogenase A (LDH-A).\(^{126}\)
4.4. Biosynthesis and cancer

In order to keep up with the rapid cell proliferation, tumor cells increase the rate of metabolic reactions to generate amino acids, nucleotides and lipids that are needed to create new biomass.\(^{112,122}\) In tumor cells the PPP pathway activity is increased. The higher activity of this pathway results not only in increased nucleotide biosynthesis but also enhances the antioxidant capacity of the cell, due to generation of NADPH, and thus protects cells against chemotherapeutics and a harmful microenvironment.\(^{120,122,127}\)

Cancer requires an enormous supply of lipids for membrane biogenesis and protein modifications. To meet this requirement cancer cells undergo major changes in their lipid metabolism and shift towards de novo lipid synthesis.\(^{112,114,137,138}\) Increased fatty acid synthesis is found in 20% to 90% of tumors and is reflected in the up-regulation of key enzymes (FASN, ACC and ACL) involved in this pathway.\(^{139-143}\) In cancer multiple oncogenic mutations leads to a rapid fatty acid synthesis i.e. mutations in the phosphatidylinositol 3 kinase (PI3K)/Akt/mTOR pathway. Activating mutations of PI3K or elimination of negative regulator PTEN results in the overexpression of SREBs.\(^{114,139,144}\) Overexpression of mTOR results in an increase in surface expression of glucose transporters.\(^{145,146}\) The breakdown of lipids by β-oxidation is suppressed by PI3K/AKT.\(^{147}\) In hematopoetic cells PI3K/AKT suppresses carnitine palmitoyltransferase-1A (CPT1A). CPT1A is the rate-limiting enzyme for β-oxidation.\(^{148}\) To fulfill the need of unsaturated fatty acids SCD-1 is overexpressed in several tumors.\(^{149}\) These enzymes and genes mentioned above, have been proposed as novel targets for tumor treatment.\(^{138,150}\)

Despite the growing evidence demonstrating deregulated fatty acid metabolism as a feature of cancer, the exact role of these metabolic alterations in the development and maintenance of the disease is not fully understood. Besides being needed for membrane biosynthesis, energy rich lipids could serve as a source of energy when nutrients are low. It has also been suggested that lipid biogenesis could play a more active role in cell transformation and cancer development. (reviewed in \(^{151}\)) Lipids acts as signaling molecules in cancer, i.e. phosphatidylinositol (3,4,5)-trisphosphate (PIP\(_3\)). PIP\(_3\) is produced by PI3K pathway in response to growth factor signaling and mediates the recruitment and activation of Akt.\(^{151}\) Lipids also play a role in the tumor microenviroment. Activation of SREBP and induction of enzymes of the mevalonate pathway are involved in the disruption of normal tissue architecture. Lipids are also involved in the interaction of cancer cells with components of the tumor stroma. For example, cancer-associated fibroblasts (CAFs) show increased expression of FASN.\(^{152}\) A study in ovarian cancer cells showed that they preferentially metastise to an abdominal fat pad (omentum). In this study it was found that omental adipocytes promote homing of these ovarian cancer cells through the induction of specific adipocyte derived cytokines.\(^{153}\) Taken together, it is clear that altered lipid metabolism has beneficial effects for tumors.
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5. Cardiolipin

Cardiolipin (CL) is a unique phospholipid, which is predominantly localized in the inner mitochondrial membrane (IMM) where it is synthesized from phosphatidylglycerol and cytidinediphosphate-diacylglycerol with the last step being catalyzed by the enzyme cardiolipin synthase.\(^ {154, 155} \) CL contains a glycerol backbone and four acyl chains which are highly unsaturated. These unsaturated acyl chains are necessary for the normal cellular function.\(^ {156} \) The precise mechanism of remodeling of the acyl chains is unclear, however it is known that newly synthesized CL is deacylated by a CL-specific deacetylase (Cld1).\(^ {156, 157} \)

One saturated fatty acyl chain is removed from CL by Cld1 to form monolysocardiolipin.\(^ {157} \)

To form mature CL, an unsaturated fatty acid is reacylated into monolysocardiolipin by the transacylase tafazzin (Taz1) (see figure 4).\(^ {158-160} \) Mutations in the \textit{tafazzin} gene results in a disease called Barth syndrome, an X-linked recessive childhood disorder. (MIM 302060) The characteristics of the disease are cardioskeletal myopathy, neutropenia and abnormal growth.\(^ {161, 162} \) Biochemically, Barth syndrome is characterized by decreased levels of CL and increases in monolysocardiolipin\(^ {163, 164} \) and a shift is observed in the level of unsaturation of the acyl side chains.\(^ {164} \) In addition, the CL species in Barth syndrome cells are more saturated than the CL species in control cells.\(^ {164} \)

Phospholipids are the main component of membranes and are important for maintaining the structural integrity of membranes. CL has several functions in the mitochondria. CL is required for optimal activity of complex I (NADH: ubiquinone oxidoreductase), complex III (ubiquinone: cytochrome c oxidoreductase), complex IV (cytochrome c oxidase) and complex V (ATP synthase).\(^ {165} \) CL is associated with and modulates the activity of several enzymes involved in the respiratory chain, including cytochrome c oxidase, carnitine palmitoyltransferase, creatinephosphokinase, the pyruvate translocator, the phosphate transporter, mono-, di-, and tricarboxylate carriers, glycerol-3-phosphate dehydrogenase, ATP/ADP translocase and ATP synthase.\(^ {154, 166, 167} \)

Next to its role in normal mitochondrial physiology, CL has also been shown to associate with members of the apoptotic machinery, including cytochrome c, Bid and caspase 8.\(^ {165} \) Cytochrome c interacts with CL in the outer leaflet of the IMM through two independent binding sites.\(^ {168-171} \) Kagan et al. found that CL-bound cytochrome c acts as a peroxidase capable of catalyzing H$_2$O$_2$-dependent peroxidation of CL and that this CL oxidation is an essential step in the release of cytochrome c during apoptosis.\(^ {172} \) During apoptosis, Bid is cleaved by caspase 8 to produce tBid. tBid translocates to the mitochondria and binds to CL-enriched contact sites and induces the translocation of BAK and BAX to the mitochondrial outer membrane.\(^ {154, 173} \) In Fas receptor activated apoptosis, CL provides an anchor and activating platform for caspase 8 translocation to the mitochondria.\(^ {174} \) CL is also necessary for the function and stabilization of the caspase-8/BID complex.\(^ {175} \) The translocation of CL from IMM to the contact sites is mediated by phospholipid scramblase.
Mitofusin-1 interacts with OPA1, which controls the tight structure of the mitochondrial cristae, keeping most cytochrome c in the cristae. tBid formation also results in the disruption of OPA1-mediated tight structure of the cristae resulting in the release of cytochrome c into the intermembrane space and thereby facilitates its release from mitochondria when these are permeabilized.\(^\text{(154)}\)

**Figure 4: Cardiolipin synthesis and remodeling in eukaryotes.** In cardiolipin synthesis, phosphatidic acid is converted into cytidinediphosphate-diacylglycerol (CDP-DAG) by CDP-DAG synthase (CDS). CDP-DAG is converted by phosphatidylglycerolphosphate (PG-P) synthase (PGPS) to PG-P. Dephosphorylation of PG-P by a phosphatase results in phosphatidylglycerol (PG). In the final step of CL biosynthesis, condensation of one molecule of PG and one molecule of CDP-DAG by CL synthase (CS) results in the formation of immature CL. Remodeling occurs via Taffazin where phospholipase A\(_2\) (PLA\(_2\)) hydrolysis an acyl chain from immature cardiolipin to generate monolysocardiolipin (MLCL). Taffazin reacylates MLCL to generate mature and remodeled cardiolipin. Cardiolipin is degraded by hydrolysation by PLA\(_2\) via MLCL and dilyso-cardiolipin (DLCL).

6. Cancer therapeutics: history and trends

Cancer therapy was initially mainly focused on targeting DNA integrity and/or replication of DNA, or on blocking mitosis by interfering with microtubule dynamics of the mitotic spindle.\(^\text{(177)}\) An example of these types of drugs are the platinum based derivatives (i.e cisplatin), topoisomerase inhibitors (i.e irinotecan), vinca alkaloids (i.e vinblastine) and taxanes (i.e taxol). These chemotherapeutics are still widely used in the clinic today. Unfortunately, in a lot of instances resistance occurs.\(^\text{(178)}\) For instance, in the case of cisplatin DNA damage is followed by apoptosis, which can be counteracted by a multitude of resistance mechanisms that can for instance arise as a consequence of intracellular changes.
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that either effectively repair cisplatin-induced DNA-adduct, prevent DNA damage signals from activating the apoptotic machinery, or prevent cisplatin uptake. Mechanisms that interfere with DNA damage signal to the apoptotic machinery include loss of damage recognition, loss of p53 function, overexpression of HER-2/neu, activation of the PI3-K/Akt pathway, overexpression of anti-apoptotic proteins, and interference in caspase activation. Another downside of these agents are that they block DNA replication and cell division, thereby killing all cells that are rapidly dividing, not only cancer cells.

The growing insight into the hallmarks of cancer and several important technical developments like the next generation DNA sequencing techniques and advances in pharmaceutical drug discovery have contributed to the search for therapies specifically targeting these hallmarks, the so-called targeted therapies. Some cellular targets are genetically altered in cancer cells and are essential to tumor development and survival. This phenomenon has been coined oncogene addiction. Other targets are not genetically altered, but still their expression is more important in cancer cells than in normal cells, which is known as non-oncogene addiction. For an overview of different targeted therapies see table 1.

A well-known prototype and the first clinically approved targeted drug is imatinib (Gleevec, Novartis), a small molecule inhibiting the Abelson tyrosine kinase (ABL) that is translocated from chromosome 9 to the breaking point cluster region (BCR) gene on chromosome 22, forming an oncogenic BCR-ABL gene fusion, which is found in chronic myeloid leukemia (CML). Imatinib is well tolerated as chronic therapy and induces molecular remission in chronic CML. Initially, the dramatic molecular and clinical effects of imatinib raised the hope that this drug would be curative for most treated patients, however soon it became apparent that therapy resistance to this inhibitor is frequent owing to mutations in the BCR-ABL kinase domain.

There is growing evidence that tumors can escape hallmark-targeting therapy. For example, a targeted therapeutic agent inhibiting one key pathway in a tumor may not completely block a hallmark, allowing some tumor cells to survive with residual function. These cells or their progeny will eventually adapt to the selective pressure established by the therapy applied. The adaptations can be accomplished by mutation, epigenetic changes or remodeling of the microenvironment resulting in renewed tumor growth and clinical relapse. In response to targeted therapy, cancer cells can reduce their dependence on one hallmark, becoming more dependent on another. This phenomenon was for instance shown by the unexpected responses to antiangiogenic therapies. Folkman and Kalluri anticipated that effective inhibition of angiogenesis would lead to dormant tumors and maybe lead to their dissolution. However, the clinical responses to antiangiogenic drugs have been found to be transient. Clinical validation for this evasive resistance is shown by the increased invasion and local metastasis observed in glioblastomas treated with
antiangiogenic therapies. These acquired resistance to therapies targeting only one hallmark are an obstacle in cancer therapy therefore cotargeting of multiple core and emerging hallmarks will result in more effective therapies.

6.1. Synthetic lethality

To overcome resistance and to specifically target cancer cells but not normal cells, the exploitation of the concept of synthetic lethality has shown to be of great potential. Synthetic lethality is derived from classical genetic studies and is based on the interaction of two genes that both contribute to an essential process. When either gene is mutated alone, the cell is viable, however the combination of mutations in both genes results in cell death. This process is referred to as synthetic lethality as it is not possible to directly isolate such cells as they die instantly when they have both mutations. In cancer therapy this synthetic lethality is being explored by developing therapies inhibiting a specific gene product that is the synthetic lethal partner of the cancer related mutation. Not all oncogenes are targetable by pharmacological intervention, thus synthetic lethality is an option for these undruggable targets. On top of that, synthetic lethal therapeutic agents can be applied alone by monotherapy, or in combination with more conventional treatments. Therapies that target synthetic lethal partners of mutations in cancers cells are cancer specific, resulting in less off-target side effects. Examples of this method is PARP1 inhibition in BRCA1/2 mutant tumors. BRCA1/2 are important in DNA repair of double strand breaks, PARP1 is an enzyme involved in single strand break repair. Inhibition of PARP1 results in an accumulation of DNA breaks which are not repaired resulting in cell death.

An example that looks like synthetic lethality is targeting the RAF-MEK pathway in KRAS mutant tumors. Combination of RAF inhibition together with MEK inhibition results in synthetic lethality of colorectal cancer and lung cancer cells that have a KRAS mutation. They found that inhibition of MEK increases the GTP-bound fraction of KRAS, promotes the formation of RAF1-BRAF heterodimers and drives constitutive phosphorylation of ERK. Inhibition of RAF1 therefore results in a block in the phosphorylation of ERK. These results are in accordance with a report showing that RAF1 knockdown enhanced MEK inhibition in a KRAS mutant model.

Synthetic lethality is a promising treatment mechanism, which could target tumors very effectively with less off-target effects. Currently with all these targeted therapies and synthetic lethality approaches researchers are doing their best to find a cure for cancer. However, still a lot of therapies fail to eradicate the tumor. This is suggested to be a cause of so-called cancer stem cells. This will be discussed below.
# Table 1: Therapeutic Targeting of the Hallmarks of Cancer.

<table>
<thead>
<tr>
<th>Cancer Hallmark</th>
<th>Targeted therapy (example)</th>
<th>Cancer type</th>
<th>Drug name</th>
<th>Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sustaining proliferative signaling</td>
<td>Tyrosine kinase inhibitors</td>
<td>Colorectal cancer, Lung cancer, Chronic myeloid leukemia</td>
<td>Cetuximab, Erlotinib, Imatinib</td>
<td>Yes (^{(183, 190-192)})</td>
</tr>
<tr>
<td>Evading apoptosis</td>
<td>Cyclin-dependent kinase inhibitors</td>
<td>Chronic lymphocytic leukemia, lung cancer</td>
<td>Alvocidib, Seliciclib</td>
<td>n.o (^{(193)})</td>
</tr>
<tr>
<td>Avoiding immune destruction</td>
<td>Immune activating mAb</td>
<td>Melanoma, non-Hodgkin lymphoma</td>
<td>Tremelimumab, Rituximab</td>
<td>n.o, yes (Rituximab)(^{19} ) 4, 195)</td>
</tr>
<tr>
<td>Enabling replicative immorality</td>
<td>Telomerase inhibitors</td>
<td>Multiple myeloma, myelofibrosis</td>
<td>Imetalstat</td>
<td>n.o (Clinical trials.gov)</td>
</tr>
<tr>
<td>Tumor promoting inflammation</td>
<td>Selective anti-inflammatory drugs</td>
<td>Familial adenomatous polyps</td>
<td>Celecoxib</td>
<td>n.o (^{(196, 197)})</td>
</tr>
<tr>
<td>Activating invasion &amp; metastasis</td>
<td>Inhibitors of HGF/c-Met</td>
<td>Lung cancer, medullary thyroid cancer</td>
<td>Cabozantinib, Foretinib, Tivatinib</td>
<td>n.o ((^{(198)}) and Clinical trial.gov)</td>
</tr>
<tr>
<td>Inducing angiogenesis</td>
<td>Inhibitors of VEGF signaling</td>
<td>Metastatic cancers (colon, lung,ovarian)</td>
<td>Bevacizumab</td>
<td>Yes (^{(199)})</td>
</tr>
<tr>
<td>Genome instability &amp; mutation</td>
<td>PARP inhibitors</td>
<td>Ovarian, breast, prostate</td>
<td>Olaparib</td>
<td>n.o (^{(200)})</td>
</tr>
<tr>
<td>Resisting cell death</td>
<td>Proapoptotic BH3 mimetics</td>
<td>Acute myeloid leukemia, chronic lymphocytic leukemia</td>
<td>ABT-199, ABT-263</td>
<td>n.o ((^{(201)}) and Clinical trial.gov)</td>
</tr>
<tr>
<td>Deregulating cellular energetics</td>
<td>Aerobic glycolysis inhibitors</td>
<td>Pre-clinical</td>
<td>3-bromopyruvate</td>
<td>n.o (^{(202)})</td>
</tr>
</tbody>
</table>

Abbreviations: mAb, monoclonal antibody; n.o, not observed. This list only represents a small part of the therapeutics currently used or under investigation.
7. Tumor heterogeneity and cancer stem cells

Intra-tumor heterogeneity arise among cancer cells within the same tumor as a consequence of genetic changes, environmental differences, epigenetic changes and reversible changes in cell properties.\(^{[213]}\) Two main conceptual frameworks have been elaborated to conceptualize the link between intra-tumor heterogeneity and therapy resistance. The first, and most established idea, is clonal evolution, where the tumor arises from a single mutated cell and over time acquires additional mutations resulting in subpopulations with evolutionary advantages.\(^{[214]}\) The other one is the cancer stem-cell model. This model hypothesizes that cancers are organized into a hierarchy of subpopulations of tumorigenic cancer stem cells (CSCs) and their non-tumorigenic progeny.\(^{[213, 215-217]}\) CSCs are defined as a subset of tumor cells which possess self-renewal and multi-lineage differentiation potential\(^{[218-220]}\) CSCs can be identified by various markers\(^{[219]}\), and in vivo experiments have shown that CSCs very efficiently form tumors that resemble the original tumor from which the CSCs were derived.\(^{[221]}\) It has been suggested that CSCs are responsible for recurrences and metastasis\(^{[219]}\), because they are thought to be therapy resistant (both chemo- and radiotherapy) and to effectively repopulate the tumor.\(^{[4, 221, 222]}\)

Currently, a lot of research is being done on targeting CSCs and deplete the tumor. However, up to date no specific CSCs targeted therapy is available. Therefore, drugs attacking cancer cells more broadly may be usable across multiple cancers and CSCs. This possible new approach consists of compounds that are not per se cancer-specific but work selective against cancer due to alterations in metabolism and phenotype of cancer cells as compared to normal cells. Currently, several natural compounds are intensively investigated as potential cancer drugs in this perspective.

8. Natural compounds

A significant percentage of golden standard chemotherapeutic agents are based on compounds from nature. Currently there are several classes of natural compounds or their semi-synthetic derivatives used in the clinic like vinca alkaloids, taxanes and anthracyclines.\(^{[223-228]}\) Vinca alkaloids were introduced into the clinics in late 1950s as Velban\(^{\circ}\) and Oncovin\(^{\circ}\), and later on Eldisine\(^{\circ}\) and Navelbine\(^{\circ}\) followed.\(^{[223]}\) The mechanism of action of these compounds has been identified as the destabilization of microtubules, which leads to G2/M cell cycle arrest and apoptosis.\(^{[229]}\) Taxanes belong to the terpenoids and one well known compound is Taxol (from Taxus)\(^{[225]}\), which was shown in 1979 to stabilize microtubule assembly.\(^{[226]}\) It is used in the clinic for the treatment of various cancer types, including ovarian, lung and breast cancer.\(^{[227]}\) Anthracyclines are DNA-intercalating agents that block cell division and they are derived from bacteria (Streptomyces). Doxorubicin is one of the best known members of this family and is used in the clinic for treatment of several cancer types.\(^{[228, 230]}\)
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Terpenoids (containing five-carbon isoprene units) of which the triterpenoids (containing six-carbon isoprene units; e.g. plant sterols) are a subclass\(^{231}\) can be isolated from many different plant sources and occur in different variations.\(^{232}\) Several of these variations or their synthetic derivatives are investigated as potential medicinal products against various diseases including cancer.\(^{233}\) One of the most promising triterpenoids with anti-cancer activity is Betulinic acid (BetA, 3b-hydroxy-lup-20(29)-en-28-oic acid, C\(_{30}\)H\(_{48}\)O\(_3\)), which is discussed extensively below.

8.1. Betulinic acid

BetA is a pentacyclic triterpene of the lupane type that can be isolated from various plant sources in small amounts. Its precursor betuline, which can be easily converted by oxidation into BetA, is available in high abundance from the bark of the white birch (*Betula pubescens*). Betulinic acid was for the first time isolated in 1948 from the bark of the London plane tree (*Platanus acerifolia*) by Bruckner.\(^{234}\)

8.2. Betulinic acid: pharmaceutical properties

Different pharmaceutical properties have been attributed to BetA. In particular its capacity to suppress HIV infection has attracted much attention. In 1994 it was shown for the first time that BetA has an potent anti-HIV activity.\(^{235}\) The wish to still optimize its anti-HIV activity together with intellectual property issues, has led to the synthesis of various BetA derivatives. Improved activity against HIV-1\(^{236, 237}\), and HIV-2\(^{238}\) of several BetA derivatives has been documented. Especially BetA derivative PA-457 has been preclinically developed as HIV-1 maturation inhibitor that specifically inhibit the last step in processing of Gag.\(^{237}\)

Under the name Bevirimat this compound has been tested as HIV-1 drug in the clinic in phase I/II clinical trials as single dose administration with no Bevirimat resistance mutations occurring in this study.\(^{239}\) Unfortunately, other studies showed that mutations in Gag occur and result in resistance to the compound.\(^{240-243}\) Optimization of BetA derivatives against HIV-1 and HIV-2 and Bevirimat is still ongoing with reported positive results.\(^{244-246}\)

Besides its anti- HIV activity, BetA and its derivatives have been described to have therapeutic activity against inflammation\(^{247, 248}\) and malaria.\(^{249-252}\) Anti-angiogenic\(^{253-256}\), and anti-fibrotic activity by BetA has also been reported.\(^{257}\)
8.3 Anti-cancer activity of Betulinic acid

In 1995, BetA was selected out of 2500 plant extracts in a screen by the National Cancer Institute as it displayed potent in vitro cytotoxic effects against human melanoma cells. Follow-up studies showed the first in vivo efficacy of BetA against melanoma.\(^{(258)}\)

Subsequently, it was shown that BetA is also effective against other tumor types like brain tumors, including the neuroectodermal glioma, medulloblastoma and glioblastoma and showed no toxicity in murine non-malignant neuronal cells.\(^{(259-262)}\)

In 2001, anti-proliferative effects of BetA in non-small and small cell lung cancer, ovarian carcinoma, cervix carcinoma were observed, while in contrast, peripheral blood lymphocytes and human normal dermal fibroblast were not affected by BetA, suggesting that BetA has a tumor specific effect.\(^{(263)}\)

Two years later, head and neck squamous cellular carcinoma cells were also shown to be sensitive to BetA\(^{(264)}\) Until 2007 no research had been performed on the efficacy of BetA in prevalent solid tumor types such as breast-, lung-, colon- and prostate cancer. In a study conducted by our group, using three different assays, all tested cell lines from solid tumors were sensitive to BetA: 10 lung cancer cell lines, 10 colorectal cancer cell lines, 9 breast cancer cell lines, 4 prostate cancer cell lines and 3 cervix carcinoma cell lines were strongly affected in their growth potential and viability after BetA treatment.\(^{(265)}\) BetA was shown to be a very potent agent against tumor with EC\(_{50}\) values around 10µg/ml in lung cancer cell lines, prostate cancer cell lines, cervical cancer cell lines and values ranging from 4-16 µg/ml in colorectal cancer cell lines and breast cancer cell lines.\(^{(265)}\)

In this study it was also shown that BetA even in high doses has no effect on healthy cells such as human blood-derived PBMC, cytotoxic T lymphocyte clones and activated B cells.\(^{(265)}\) The efficacy of BetA was also shown in hepatoblastoma\(^{(266)}\), rhabdomyosarcoma\(^{(267)}\) and nasopharyngeal carcinoma\(^{(268)}\) using several concentrations of BetA ranging up to 20 µg/ml. BetA has also been shown to be effective in hematological malignancies i.e BetA induced cell death in murine leukemia
cell line L1210 and in leukemia cells form patients and cell lines. The last study showed cell death inducing capacities of BetA independent of risk stratification, age and sex of the patient and leukemia type. BetA also induced cell death in human CML cell line K-562. Combined these studies indicate that BetA is a promising anti-cancer compound because of its potent and broad tumor selective activity.

8.4. Mechanism of Betulinic acid

Thus far, the precise mechanism of anti-cancer action of BetA has not been identified. Although various and broad intra cellular anti-cancer effects of the compound have been revealed until now, these effects were difficult to be linked with unique and defined molecular target(s). However, accumulating research of various research groups has revealed important characteristics of BetA-induced cancer cell death.

8.4.1. Betulinic acid and apoptosis

In 1997 Fulda et al. showed that in SHEP neuroblastoma cells apoptosis was induced after BetA treatment and that overexpression of BCL-2 and BCL-XL blocked BetA-induced loss of mitochondrial membrane potential, PARP cleavage, caspase processing and ROS production. Subsequently, in isolated mitochondria of SHEP cells, BetA triggered permeability transition (PT) and cytochrome c release, while mitochondria derived from SHEP cells that overexpressed BCL-2 or BCL-XL were resistant to BetA. Consistent with these data, BetA induced ROS production, which was inhibited by BCL-2 overexpression or antioxidants.

Although BCL-2 overexpression showed protection against BetA-induced apoptosis in SHEP and glioma cells, it only partially protected Jurkat cells, MCF-7 cells and melanoma cells against BetA. These results suggest that the protective effects of anti-apoptotic BCL-2 family members are possibly cell type dependent. In 2009, our group showed that BetA induced cytochrome c release and apoptosis in BAK/BAX double deficient mouse embryonic fibroblasts (MEFs) and in HCT116 double knock out BAK/BAX cells. The levels of apoptosis induced in the double deficient cells were comparable with the wild-type control cells, suggesting that BetA induces cytochrome c release and apoptosis independent of BAK/BAX. Our study also showed that BetA-induced cell death could be blocked by the addition of cyclosporine A, a PT-pore inhibitor suggesting an important role for this pore in BetA-induced cell death.

Interestingly, our group showed that BetA induced cell death in the presence of pan-caspase inhibitor zVAD.fmk in Jurkat cells, and at the same time PARP processing and DNA fragmentation were completely blocked. These results suggest that the cytotoxic effects of BetA are not completely caspase dependent and that other cell death pathways are likely involved.
8.4.2. Betulinic acid and autophagic cell death

The focus in unraveling the mechanism of BetA has been mainly on apoptosis. However in 2012 a study of Gonzalez et al. showed that treatment with a glycosylated derivative of BetA, named B10, leads to autophagic cell death. In this work it was shown that downregulation of autophagy genes ATG7, ATG5 and BECN1 by RNAi-mediated suppression significantly decreased B10-induced cell death. B10 induces autophagy and disrupts autophagic flux as measured by LC3 lipidation.\(^{(275)}\) Subsequently, by another research group it was shown that BetA induces autophagy in multiple myeloma cells by an induction of LC3 lipidation. However a block in autophagic flux was observed as P62 levels were also increased after BetA treatment.\(^{(276)}\) The main conclusion of the latter study was that BetA inhibits autophagic flux and the compound induced cell death via apoptosis. These two studies, one using a derivative of BetA, are the only studies described.

With these contradicting results and as above mentioned the role of autophagy in the induction of cell death remains unclear. Therefore more research about the role of BetA in autophagic cell death is needed. In this thesis we investigated the role of autophagy as a cell death mechanism (chapter 2)

8.5. Betulinic acid in combination therapy

Several studies have described the effect of BetA in combination with another therapeutic intervention. In 2000, it was shown that the combination of BetA with radiotherapy had an additive effect in human melanoma cells.\(^{(277)}\) BetA improved vincristine-induced cytotoxicity in mouse melanoma cells and reduced the number of metastases in vivo.\(^{(278)}\) Fulda et al. showed that combination of BetA and anticancer drugs cisplatin or doxorubicin cooperated to induce apoptosis and to inhibit clonogenic survival of tumor cells.\(^{(279)}\) It was also shown that BetA sensitizes drug-resistant colon cancer cells and esophageal squamous carcinoma cells to oxaliplatin, irinotecan and 5-fluorouracil.\(^{(280, 281)}\) The combination of BetA and docetaxel or 2-methoxyestradiol resulted in increased apoptosis.\(^{(282)}\) A study by the group of Vordermark showed that an additive effect of BetA and radiotherapy was also observed in glioma cells. This effect was most pronounced when cells were hypoxic.\(^{(283)}\) On top of that it was shown that in pancreatic cancer the combination of BetA and mithramycin A (a DNA-binding, anti-tumor and neuroprotective antibiotic originally isolated from S. grieseus) resulted in an inhibitory effect on cell proliferation, invasion and angiogenesis.\(^{(284)}\) This combination also resulted in less discernible side effects than gemcitabine alone, which was used as a standard reference drug.\(^{(284)}\) Together these data indicate that BetA is a very potent compounds for combination treatments.
8.6. Betulinic acid in vivo

Besides many in vitro studies, BetA has also been shown to be effective in vivo. Because of the lipophilic characteristics of BetA and its consequently poor solubility in vivo studies has been limited. The first study describing in vivo application of BetA used athymic mice with human melanomas xenografted subcutaneously that were treated by intraperitoneal injections of BetA in a formulation with polyvinylpyrrolidone (PVP). A dose of 50 mg/kg body weight was injected every four days for six times resulting in tumor regression. In this study no toxicity as measured by weight loss was observed up to 500 mg/kg body weight. As described before BetA and in combination with vincristine reduced the number of metastases. The treatment dose of BetA (dissolved in DMSO) was 10 mg/kg divided over 10 days out of 17 days. BetA dissolved in DMSO was also used in breast cancer xenograft model where mice were treated intraperitoneal with 50 and 100 mg/kg bodyweight BetA every 3-4 days for 6 doses resulting in delayed tumor growth. Another study, using human ovarian carcinoma xenograft model showed a clear survival benefit for mice treated with intraperitoneal injections of a formulation of ethanol, Tween-80 and water (10%, 10% and 80%). Mice were injected every 3-4 days with 100 mg/kg bodyweight for a total of six injections. Oral treatment with 10 or 20 mg/kg bodyweight BetA in corn oil in mice bearing subcutaneously grafted prostate cancer cell line resulted in inhibition of tumor outgrowth as compared to corn oil alone. Also intravenous injection of BetA (10 mg/kg bodyweight, every day for 14 days) has been shown to reduce adenomacarcinoma xenograft size. All these studies showed no systemic signs of toxicity, however the formulations used are either not suitable for human application or are not precisely defined and therefore cannot be standardized. Therefore our group investigated liposomes as a delivery system of BetA. Liposomes are small vesicles consisting of one or more concentric phospholipid bilayers with an aqueous core. Both water soluble as well as lipophilic compounds can be incorporated in the liposomes. This study showed that intravenous injection of liposomes containing BetA both reduced tumor growth and prolongs survival of colon carcinoma and lung carcinoma bearing mice. Mice were treated three times a week with 50mg/kg bodyweight for three months (lung carcinoma) and two months (colon carcinoma). This study also showed that oral administration of BetA liposomes reduces tumor growth and prolongs survival of colon carcinoma bearing mice, although these data were less pronounced as intravenous injections. In this study no systemic toxicity was observed, suggesting that BetA-liposome formulation is a possible formulation for BetA to be used in preclinical studies.
Outline of this thesis

The scope of this thesis was to investigate the mechanisms by which BetA induces cell death in cancer cells in more detail. At the start of the studies described in this thesis several questions urgently needed an answer. Although BetA induces cell death via apoptosis, when blocking this form of cell death cancer cells still die.\(^{(265)}\) In tumor therapy it is still unclear whether activation of autophagy contributes to cell death or rather represents a resistance/survival mechanism. We therefore investigated the roles of other cell death mechanisms including necroptosis and autophagy in BetA-induced cell death. The results of this investigation are described in chapter 2. BetA induces cell death independently of BAK/BAX but in a mitochondrial dependent fashion, but no reports exist revealing how this form of cell death occurs, we set off to study the mechanism by which BetA induces cell death in more detail. We found that BetA interferes with lipid cell metabolism. The results of this research are described in chapter 3. Cancer stem cells are tumor resistant and currently no effective therapy has been shown for these cells. BetA is a very potent and broad acting compound therefore we treated colon cancer stem cells with BetA. Results of these experiments are laid out in chapter 4. Finally, we were also interested in the effect of BetA on several lipids in the cell. We set-up a lipidomics pipeline and used a BetA dataset to test the functionality of this pipeline as well as to investigate the difference in lipids after BetA treatment. Chapter 5 contains these results. In chapter 6 all data of this thesis are discussed in relation to the literature.
Chapter 1

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Chapter 2

Betulinic acid-induced mitochondria-dependent cell death is counter balanced by an autophagic salvage response

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"Tut, tut, child!" said the Duchess. "Every thing's got a moral, if only you can find it."

Lewis Carroll, Alice in Wonderland
Chapter 2

Abstract

Betulinic acid (BetA) is a plant-derived pentacyclic triterpenoid that exerts potent anti-cancer effects in vitro and in vivo. It was shown to induce apoptosis via a direct effect on mitochondria. This is largely independent of proapoptotic BAK and BAX, but can be inhibited by cyclosporin A (CsA), an inhibitor of the permeability transition (PT) pore. Here we show that blocking apoptosis with general caspase inhibitors did not prevent cell death, indicating that alternative, caspase-independent cell death pathways were activated. BetA did not induce necroptosis, but we observed a strong induction of autophagy in several cancer cell lines. Autophagy was functional as shown by enhanced flux and degradation of long-lived proteins. BetA-induced autophagy could be blocked, just like apoptosis, with CsA, suggesting that autophagy is activated as a response to the mitochondrial damage inflicted by BetA. As both a survival and cell death role have been attributed to autophagy, autophagy-deficient tumor cells and mouse embryo fibroblasts were analyzed to determine the role of autophagy in BetA-induced cell death. This clearly established BetA-induced autophagy as a survival mechanism and indicates that BetA utilizes an as yet-undefined mechanism to kill cancer cells.
Betulinic acid (BetA) is a naturally occurring triterpenoid with potent cytotoxic effects on cancer cells.\textsuperscript{1–4} It was initially proposed to have a direct effect on the mitochondria and to induce apoptosis in a BCL-2-dependent fashion.\textsuperscript{5–7} However, BCL-2 overexpression can only provide short-term protection and eventually these cells do succumb to apoptosis. In addition, BAK/BAX double deficiency does not protect against BetA-induced cell death,\textsuperscript{8} indicating that cell death ensues independently of the BCL-2 family. Mitochondrial outer membrane permeabilization (MOMP), however, is crucial as cyclosporin A (CsA) and bongkrekic acid, both inhibitors of the permeability transition pore (PT-pore), and as a consequence MOMP were able to protect BetA-treated cells from releasing cytochrome c and subsequent apoptosis.\textsuperscript{7,8} Downstream of the mitochondria caspases are activated, but direct inhibition of caspase activity with the pan-caspase inhibitor zVAD.fmk (N-benzyloxy carbonyl-Val-Ala-Asp-fluoromethylketone) did not offer protection to BetA-induced cell death.\textsuperscript{4} Combined this indicates that BetA does induce apoptosis, but that additional mechanisms must exist by which cell death is induced by the compound.

Alternative cell death pathways include necrosis, necroptosis, lysosomal membrane permeabilization (LMP) and autophagy. Necrosis is a form of passive cell death that is induced upon strong insults such as mechanical injury of cells. Typical features include swelling, rupture of organelle membranes as well as the outer cell membrane and as a result the cell contents are released, often causing inflammation in vivo.\textsuperscript{9} Necroptosis, a highly regulated form of necrosis, can be induced by ligation of death receptors in the presence of caspase inhibitors.\textsuperscript{10} Necroptosis is regulated by distinct complexes called the necrosome or ripoptosome that include receptor interacting protein (RIP) kinases, FAS-associated death domain and caspase-8.\textsuperscript{10,11} Necroptosis can be blocked by small molecules, such as necrostatin-1, that allosterically block the kinase activity of RIP1.\textsuperscript{12,13}

Lysosomal cell death is induced by a destabilization of the lysosomal membrane.\textsuperscript{14} The cell death depends on the leakage of lysosomal components into the cytoplasm, and the activity of cathepsins that can induce a necrotic-like or apoptotic cell death depending on the extent of the leakage. As such the induction of cell death is clearly distinct from necrosis and apoptosis; nevertheless, the execution phase is similar.

Autophagy is a highly conserved cellular proteolytic degradation process in which cytosolic components are first encapsulated by a membrane and subsequently degraded in the lysosome, thereby providing new building blocks for the cell. Autophagy is typically induced upon nutrient starvation. However, autophagy also involves sequestration of damaged cytoplasmic components and organelles, via induction of de novo double-membrane vesicles (autophagosomes) that surround the cargo. The autophagosome transports the
Chapter 2

cargo to the lysosome where fusion of the autophagosome and lysosome leads to degradation of the cargo (autophagic flux). Autophagy, even though initially regarded as a cell survival mechanism, has also been suggested to serve a role in inducing cell death. It may therefore represent a balancing mechanism between cell survival and cell death. Autophagy can target cytosolic components specific for degradation, known as specific autophagy. Specific autophagy can include ubiquitinated proteins, peroxisomes and mitochondria. Mitophagy involves the selective degradation of mitochondria and is among others used for clearance of damaged mitochondria. 

Previously, BetA and a derivative of BetA, B10, have been shown to induce autophagosome formation in multiple myeloma cells and glioblastoma cells. In these studies it was suggested that the autophagic flux was prevented, leading to an accumulation of undigested autophagosomes. Although both studies observed autophagy, the role of autophagy as a cell death mechanism was not addressed for BetA.

We hypothesized that autophagy/mitophagy is induced upon BetA treatment to clear the damaged mitochondria. We show that autophagy is massively induced in various BetA-treated tumor cells, but is prevented by CsA, suggesting that autophagy occurs downstream of the BetA-induced mitochondrial damage. With the use of knockout and knockdown studies of key regulators of the autophagy pathway, we demonstrate that autophagy serves as a rescue pathway and is not responsible for the cell death induced by BetA.

Results

BetA-induced cell death is independent of apoptosis.

BetA induced a very potent form of cell death in HeLa cells that displayed features like cell membrane rupture (Figure 1a), apoptosis (Figures 1b and c) and mitochondrial depolarization (Figures 1d and e). The effect of BetA was, as shown before, concentration dependent, starting at 7.5 µg/ml and reaching a plateau at 10 µg/ml (Supplementary Figure 1). Previously we have shown, in Jurkat cells, that BetA-induced apoptosis is blocked by co-treatment with zVAD.fmk, but that this pan-caspase inhibitor does not prevent cell death as measured by propidium iodide (PI) exclusion. Similarly, cell death induced by BetA in HeLa cells was only partly blocked with a more potent caspase inhibitor Q-VD-OPh (Figure 1f), which did block apoptotic features like DNA fragmentation (results not shown). This indicates that caspase inhibition was effective, but not sufficient to block the induction of cell death. Thus, while apoptosis is induced by BetA, these findings indicate that alternative cell death pathways are activated as well. To study whether necroptosis serves as an alternative cell death mechanism induced by BetA, we made use of necrostatin, a specific inhibitor of this cell death mechanism. Either necrostatin alone or in combination with caspase inhibition failed to prevent cell death (Figure 1f). As a control for the action of
Betulinic acid induces a rescue autophagy response

necrostatin, U937 cells were used, which have been reported to undergo necroptosis upon tumor necrosis factor-α (TNF-α) treatment\textsuperscript{12} in the presence caspase inhibitors. In accordance with literature\textsuperscript{12} this indeed led to a significant induction necroptosis, which was prevented by necrostatin (Supplementary Figure 2). As this combination did not inhibit BetA-induced cell death, our data point to a cell death mechanism that is independent of caspases and necroptosis.

**Figure 1. BetA induces cell death independent of apoptosis and necroptosis.** HeLa cells were subjected to 10 µg/ml BetA for 48 h after which cell death by PI exclusion (a), DNA fragmentation (b), phosphatidylserine (PS) exposure (c), and loss of mitochondrial activity by mitotracker orange (d) or JC-1 (e) were assessed (f) HeLa cells were pre-treated for 1 h with or without 20 µM Q-VD-OpH and with or without 25 µM necrostatin-1 and subjected to 10 µg/ml BetA for 48 h after which cell death was assessed via PI exclusion. Mean ± S.D. of three independent experiments are shown, *P< 0.05; **P< 0.01; ***P<0.001; NS, not significant.

**BetA induces autophagy**

To determine whether autophagy is responsible for the cell death observed, LC3 conjugation was studied. Conjugated LC3 (LC3-II) is associated with autophagic membranes and therefore fusion of LC3 to green fluorescent protein (GFP) can be used to detect autophagosomes.\textsuperscript{25} In untreated cells, LC3–GFP is evenly dispersed in the cells, with a slight preference for the nucleus, whereas in cells where autophagy is induced, LC3 translocation is evident (Figure 2a).\textsuperscript{25} Both HeLa\textbackslash LC3–GFP and MCF-7\textbackslash LC3–GFP cells treated with the classical autophagy inducer rapamycin displayed a clear induction of punctuated LC3–GFP structures. Interestingly, BetA was even more potent in inducing autophagy, showing
enlarged punctae (Figure 2a). To validate that BetA indeed induced autophagy, a separate measure to monitor the induction of autophagy was used, namely the processing of LC3-I to its PE-conjugated LC3-II using immunoblotting. Rapamycin treatment resulted in enhanced LC3-II levels in HeLa\LC3–GFP, A549, MCF-7\LC3–GFP and SW480\LC3-GFP cells as compared with their DMSO-treated counterparts (Figure 2b). Analyzing multiple independent assays consistently revealed this induction, but due to the variation in background levels of LC3-II it only reached significance in HeLa and A549 cells (Figure 2c). In contrast, BetA-induced LC3-II levels are significantly elevated in all cell lines and were clearly more pronounced as compared with rapamycin (Figures 2b and c), similar to the observations with LC3–GFP staining. The effect of BetA on autophagy occurs relatively rapid upon BetA treatment and required concentrations that are also exerting a toxic effect (7.5–10 µg/ml) (Supplementary Figures 3a and b).

Both detection of LC3-II via immunoblotting and LC3–GFP translocation are measures for the amount of autophagosomes present at a certain time point, but do not provide information about the cause of this phenotype. It is possible that an increased number of autophagosomes is caused by an inhibition of basal autophagic flux rather than by induction of autophagy. Previously, it was reported that BetA in KM3 cells dose-dependently induces the expression of LC3-II, but also of p62, a protein that is normally degraded during autophagy. This study suggested that BetA rather inhibited autophagic flux instead of inducing autophagy. To analyze the effect of BetA on p62 we studied its expression in HeLa cells. This confirmed a strong induction of p62 upon BetA treatment (Supplementary Figure 3c). However, this is due to induction of de novo synthesis and could be blocked with the addition of cycloheximide. The combination of cycloheximide and BetA did reveal degradation of p62, pointing to a functional flux (Supplementary Figure 3c). To better evaluate this autophagic flux, degradation of long-lived proteins was measured, which reportedly are at least in part degraded by autophagy. In agreement, rapamycin clearly induced degradation of these long-lived proteins after 14 h (Figure 2d). Moreover, degradation continued after this time point as was evident from analyzing the release of labeled amino acids in the time period between 14 and 20 h (Figure 2e). In contrast to the finding of Yang et al. That BetA prevents autophagic flux, BetA clearly increased the degradation of long-lived proteins after 14 h and the following 6 h after medium change. This was also more pronounced as compared to rapamycin-induced degradation (Figures 2d and e). Because degradation of long-lived proteins is not solely specific for autophagy and cannot discriminate between proteasomal- and autophagosomal degradation more specific assays using tandem RFP/eGFP-tagged LC3 as well as a tandem mCherry/GFP-tagged p62 were used. As RFP and mCherry are pH stable, they remain fluorescent after fusion of the autophagosomes with the lysosomal compartment, while eGFP fluorescence is lost. This allows detection of autophagic flux by simply observing the formation of red fluorescent lysosomes from green/red fluorescent autophagosomes. Using either LC3
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(Figure 2f) or p62 (Supplementary Figure 3d) we observed that rapamycin as well as BetA induced a functional autophagic flux using. This confirms that BetA is a potent inducer of autophagy resulting in an enhanced autophagic flux.

![Image](https://example.com/image.png)

**Figure 2. BetA induces autophagy** (a) HeLa\LC3-GFP cells and MCF-7\LC3-GFP cells were treated with 10 µg/ml BetA or 10 µM rapamycin and after 18 h cells were analyzed by confocal microscopy. Quantification of LC3 puncta was performed using Image J. Mean of positive pixels/total pixel cell (%) ± S.D. of three fields of view per sample are shown. (b) HeLa\LC3-GFP, A549, MCF-7\LC3-GFP and SW480\LC3-GFP were treated with 10 µg/ml BetA or 10 µM rapamycin for 18 h. LC3 processing was assessed via immunoblotting. Results for endogenous LC3 are shown. LC3-I (18 kDa) and LC3-II (16 kDa). Tubulin is used as a control. (c) Band intensity of western blot was quantified using Image J software. DMSO control is set to 1. Mean ± S.D. of three independent experiments are shown, *P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant. (d) MCF7 cells were treated with the indicated concentrations of BetA or 1 µM rapamycin and after 14 h degradation of long-lived proteins was measured or (e) after 14 h medium was refreshed and degradation in the following 6 h was measured. Mean ± S.D. of triplicates are shown. (f) HeLa and A549 cells with tandem fluorescent LC3 (mRFP-eGFP-LC3) were treated with 10 µg/ml BetA or 10 µM rapamycin and after 18 h cells were analyzed by confocal microscopy. Quantification of red LC3 puncta was performed using Image J. Mean of positive pixels/total pixel cell (%) ± S.D. of three fields of view per sample are shown.
Cyclosporin A blocks BetA-induced autophagy.

Permeability transition pore (PT-pore) opening results in membrane depolarization and leads to cytochrome c release and subsequent caspase activation. The proposed structure of the pore is formed by a voltage-dependent anion channel, adenine nucleotide translocator and cyclophilin D complex, and opening of the PT-pore can be blocked by inhibition of cyclophilin D using CsA. Previously we have shown that BetA-induced apoptosis and cytochrome c release proceeded in a PT-pore-dependent fashion. As CsA inhibits BetA-induced apoptotic features, the effect of CsA on BetA-induced autophagy was investigated. HeLa\LC3–GFP cells were treated with BetA alone or in combination with CsA and analyzed via confocal microscopy. Autophagosome formation was clearly inhibited in the presence of CsA (Figure 3a). A similar CsA dependency was observed in MCF-7\LC3 cells (Figure 3a). The effect of CsA was confirmed by immunoblotting for LC3 in BetA-treated HeLa, but also in other cancer lines (AS49, MCF-7 and SW480). In all tested cell lines, BetA-induced formation of lipidated LC3-II was reduced when cells were pretreated with CsA (Figures 3b and c). These data suggest that BetA-induced autophagy is a consequence of mitochondrial damage triggered by BetA and can be prevented by inhibition of PT-pore opening.

Autophagy serves as a rescue pathway, not as an alternative cell death pathway in BetA-treated cells.

Autophagy, even though initially regarded as a cell survival pathway, can play a role in dying cells as well and has been suggested to serve as a balancing mechanism between cell survival and cell death. Autophagy is detectable already at low BetA concentrations (7.5 µg/ml) and we reasoned that it is primarily induced as a rescue mechanism. However, it is feasible that at higher BetA concentrations autophagy is induced beyond a certain threshold, which shifts the balance from cell survival to cell death. To test this hypothesis, we used cell lines with a deficiency in ATG5 or ATG7, which are crucial regulators in the induction of autophagy. First we used retroviral shRNA against ATG5 in HeLa and HeLa\LC3–GFP cells. ATG5 knockdown levels of 77 and 86% were obtained with shRNA against ATG5 in HeLa and HeLa\LC3–GFP cells, respectively (Supplementary Figure 4a). The abrogation of autophagy in these cells was confirmed by confocal microscopy (Figure 4a). Importantly, when analyzing BetA-induced cell death in HeLa ATG5 knockdown cells, this was found, if anything, to be enhanced as compared to the cell death induced in control knockdown cells (Figure 4b). The increase in BetA-induced cell death in autophagy-impaired cells was also observed in MCF-7\LC3–GFP cells (Supplementary Figures 4a and b). To formally confirm that impaired autophagy enhances BetA-induced cell death, MEFs derived from Atg5 or Atg7 knockout mice were used. While these cells are completely autophagy deficient, they showed increased levels of BetA-induced cell death (Figure 4c) and Annexin
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V exposure (Supplementary Figure 4c). Interestingly, caspase inhibition in these autophagy-deficient cells did not prevent cell death either (Figure 4d). However, both autophagy-proficient and −deficient cells displayed a similar increase in mitochondrial depolarization (Figure 4e), suggesting that the initial mitochondrial insults are independent of the induction of autophagy. Combined these data indicate that autophagy serves primarily as a survival mechanism in BetA-treated cells and that BetA-induced cell death is independent of autophagy, necroptosis and caspases and follows an as yet undefined pathway.

**Figure 3. CsA inhibits BetA-induced autophagy** (a) HeLa\LC3-GFP cells and MCF-7\LC3-GFP cells were pre-treated with 10 µg/ml CsA for one hour and subjected to 10 µg/ml BetA for 18 h and analyzed by confocal microscopy. Quantification of LC3 puncta was performed using Image J. Mean of positive pixels/total pixel cell (%) + S.D. of three fields of view per sample are shown. (b) HeLa\LC3-GFP, A549, MCF-7\LC3-GFP and SW480\LC3-GFP were pre-treated with 10 µg/ml CsA for one hour and subjected to 10 µg/ml BetA for 18 h. LC3 processing was assessed via immunoblotting. Results for endogenous LC3 are shown; LC3-I (18 kDa) and LC3-II (16 kDa). Tubulin is used as a control. (c) Band intensity of LC3 western blot was quantified using Image J software. DMSO control is set to 1. Mean + S.D. of three independent experiments are shown, *P < 0.05; **P < 0.01; ***P < 0.001; N.S., not significant.
Figure 4. Autophagy serves as a rescue pathway (a) HeLa\(\text{LC3-GFP cells with pSuper empty (control) or ATG5 #2 knockdown were treated with 10 µM rapamycin for 6 h and analyzed with confocal microscopy. Representative pictures of two experiments are shown. Quantification of LC3 puncta was performed using Image J software. Mean of positive pixels/total pixel cell (%) ± S.D. of three fields of view per sample are shown. (b) HeLa cells with ATG5 #2 knockdown and the control were subjected to different concentrations of BetA for 48 h after which cell death was measured via PI exclusion and DNA fragmentation was assessed. Mean ± S.D. of triplicates are shown. (c) Mouse embryonic fibroblasts lacking either ATG5 or ATG7 and their respective control cells were subjected to different concentrations of BetA and cell death (24 h) using (e) PI exclusion was measured. (d) Mouse embryonic fibroblasts lacking either ATG5 or ATG7 and their respective control cells were subjected to different concentrations of BetA for 24 h in the presence or absence of 20 µM ZVAD.fmk and cell death using PI exclusion was measured. Mean ± S.D. of three independent experiments are shown. (e) Loss of mitochondrial activity measured by JC-1 after 18 h was assessed. Mean ± S.D. of three independent experiments are shown.

Discussion

BetA is a promising anti-cancer agent with apoptosis-inducing effects that acts on the PT-pore in a BAX/BAK-independent fashion. Here we show that BetA highly efficiently induced autophagy in several cancer cells. Autophagic flux was found to be functional and led to
enhanced degradation of long-lived proteins and degradation of GFP in tandem fluorescent LC3 and p62 proteins (Figure 2 and Supplementary Figure 3). Our observations are in contrast to previous findings by Yang et al.\textsuperscript{24} reporting that BetA inhibits autophagic flux in human multiple myeloma cells as measured by accumulation of p62 protein. Similar to BetA, B10, a semi-synthetic glycosylated derivative of BetA, induced cell death in both an apoptosis-dependent and apoptosis-independent fashion. In response to B10 treatment, LMP and subsequent release of lysosomal enzymes was observed in a human glioblastoma cell line, impairing autophagy in later stages.\textsuperscript{23} LMP results in the release of lysosomal hydrolases and cathepsins into the cytosol.\textsuperscript{37} Massive lysosomal leakage leads to uncontrolled necrosis, whereas minor LMP can activate either the intrinsic caspase-dependent apoptosis pathway or caspase-independent alternative cell death programs.\textsuperscript{38–43} LMP could therefore also be a possible mechanism by which BetA induces cell death. However, in contrast to B10, which disrupts the autophagic flux, our data show that autophagy is enhanced after BetA treatment. It could well be that at later time points the flux is halted, potentially due to an overload of cargo. This could explain the relatively large LC3–GFP structures that are observed at later time points. Nevertheless, we show that the initial enhanced autophagic flux also serves a protective function as it delays the execution of cell death, while it does not appear to limit the mitochondrial depolarization. Blocking autophagy completely is detrimental to the cells confirming that the observed increase in autophagosomes and LC3-II is not simply due to a block in the flux.

An interesting connection between BetA-induced apoptosis and autophagy is the fact that both are efficiently inhibited by CsA, an inhibitor of the PT-pore\textsuperscript{8} (Figure 3) CsA has been reported to block mitophagy induced by loss of MOMP in rat hepatocytes.\textsuperscript{44,45} Blocking of apoptosis and autophagy by CsA hints to a scenario in which both pathways are triggered by a common upstream event that is related to PT-pore opening. How this is achieved by BetA remains yet to be established. It is consistent though with the observations in autophagy-deficient cells, which do show a decrease in cell death, but not in mitochondrial defects. This suggests that the primary insult is independent of autophagy, but that the execution of cell death is dampened by an initial salvage response through the induction of autophagy. The fact that autophagy occurs relatively rapid upon BetA treatment is also in line with this notion.

Even though our results suggest that autophagy does not primarily serve as a cell death mechanism, it is conceivable that it plays an indirect role in the toxicity exerted by BetA. At higher concentration of BetA, autophagy may keep the balance up for a period of time, after which either the mitochondrial damage is too massive to be counteracted by autophagy or that lysosomal exhaustion occurs, which as a consequence will prevent further autophagic flux. This may then result in release of cytochrome c, activation of caspases and subsequent apoptosis. This is supported by the observations that BetA-induced cell death is higher in
autophagy-deficient cells compared to control cells (Figure 4 and Supplementary Figure 4). Nevertheless, even under these conditions caspase inactivation is not sufficient to rescue the cells either, suggesting that the mitochondrial damage inflicted is not compatible with cellular survival. Taken together our findings show that BetA-induced cell death is independent of caspases, necroptosis and autophagy and, thus, alternative cell death mechanisms must be involved. This potentially explains the high tumoricidal efficacy of BetA, as it induces cell death that is not prevented by classical antiapoptotic mechanisms.

Materials and Methods

Chemicals/antibodies.
Betulinic acid (BioSolution Halle, Halle, Germany, (>99% purity) was dissolved in DMSO at 4 mg/ml and stored at -80 °C. PI and CsA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rapamycin was purchased from VWR (Amsterdam, The Netherlands) and C-14 valine (CFB75-50 μCi) from Amersham Biosciences (Amersham, UK). Q-VD-OPh was purchased from R&D Systems (Minneapolis, MN, USA). Necrostatin-1 and TNF-α were obtained from Enzo Life Sciences (Farmingdale, NY, USA). Anti-LC3 antibody (51520) was obtained from Abcam (Cambridge, UK). Anti-ATG5 antibody and anti- SQSTM1/p62 were from Cell Signaling Technology (Danvers, MA, USA). Anti α Tubulin antibody was from Santa Cruz Biotechnology (Dallas, TX, USA). Annexin V-APC and 7-AAD were from BD Biosciences (Franklin Lakes, NJ, USA). Mitotracker Orange CMTMRos and JC-1 were obtained from Life Technologies (Carlsbad, CA, USA).

Cell culture.
HeLa cells, A549 cells, MCF-7 cells, SW480 and U937 cells were obtained from the ATCC (Manassas, VA, USA). MCF-7 cells overexpressing LC3–GFP were generated as described.46 HeLa cells and SW480 cells overexpressing LC3-GFP were made by stable transfection of plasmid pEGFP-C1-LC3, which was a gift from G. Kroemer (Institut Gustave Roussy, Villejuif, France). All LC3–GFP overexpressing cell lines were cultured with 500 μg/ml G418 in order to select for LC3–GFP. ATG5 knockout and the corresponding control MEFs were kindly provided by N Mizushima (Department of Cell Biology, Okazaki, Japan)25 and the ATG7 knockout and corresponding control MEFs were obtained from M Komatsu (Department of Molecular Oncology, Tokyo, Japan).35 All cells were cultured in IMDM supplemented with 8% FCS, 2mM L-glutamine, 40 U/ml penicillin and 40 μg/ml streptomycin.

RNA interference.
Retroviral vectors pSuper ATG5 #2, pSuper ATG5 #3 and pSuper empty, a gift from S.W. Tait (Beatson Institute, Institute of Cancer Sciences, Glasgow, UK),47 were transfected into
Phoenix Amphotrophic cells using the standard Ca\(^{2+}\)-phosphate procedure. After 48 h, the virus containing medium was centrifuged at 1200 r.p.m. to purify virus from cell debris and supernatant was supplemented with 10 µg/ml polybrene. Target cells were plated at 70% confluence and allowed to attach overnight in standard medium. For infections, the culture medium was replaced with the virus containing medium and incubated for 8 h at 37 °C. Transduced cell populations were subsequently selected with 1–2 µg/ml puromycin, depending on cell type (2 µg/ml for HeLa cells and 1 µg/ml for MCF-7 cells). After 1 week of selection, expression of the targeted proteins was determined by western blot.

**Cell death analysis.**

Cell death was determined by PI exclusion. In short, 50 000 cells were plated in a 24-well plate and allowed to attach overnight. Cells were treated for 48 h and harvested. The cell pellet was resuspended in 200 µl medium and stained with PI at 1 µg/ml just before measuring by flow cytometry. Samples were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA). For quantification of apoptotic DNA fragmentation (Nicoletti assay) cells were resuspended in Nicoletti buffer\(^{48}\) supplemented with 50 µg/ml PI for 12 h, subsequent flow cytometric measurement of PI-stained nuclei was performed. Phosphatidylserine exposure during apoptosis was detected by annexin V assay. In short, 50 000 cells were plated in a 24-well plate and allowed to attach overnight. Cells were treated for 48 h and harvested. The cell pellet was stained with annexin V-APC and 7-AAD for 15 min at RT, subsequently flow cytometric measurement of annexin V and 7-AAD was performed.

**Mitochondrial membrane potential analysis.**

Mitochondrial activity was measured with Mitotracker Orange CMTMRos. Treated cells were incubated for 30 min with 25 nM Mitotracker Orange at 37 °C. After staining, cells were washed and 7-AAD was added followed by cytometric analysis. As a second mitochondrial membrane potential assay, JC-1 staining was used. Cells were stained for 30 min at RT with 4 µM JC-1. Depolarization was measured in FITC and PE channel by flow cytometry.

**LC3 fluorescence microscopy.**

HeLa and MCF-7 cells overexpressing LC3–GFP were cultured on Poly-D-lysine-coated 24 mm diameter glass coverslips in six-well plates and treated with BetA, rapamycin and CsA for indicated time points and concentrations. The glass coverslips were mounted into a chamber of the microscope for live-cell imaging and Z-stack measurements and placed in a Leica DMI 6000 (TCS SP8) microscope (with adaptive focus, Motorized XY-Stage and SuperZ Galvo) and a case incubator at 37 °C. Samples were analyzed using Leica Las AF software. Quantification was performed with Image J\(^{49}\) generating a cut-off for the basal dispersed LC3 fluorescence, allowing for the quantification of fluorescence in the concentrated areas (LC3–GFP-associated autophagosomes).
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Tandem fluorescence microscopy.
HeLa and A549 cells were transiently transfected with tandem fluorescent LC3 (mRFP–eGFP–LC3, kindly provided by T Yoshimori\textsuperscript{30} using polyethylenimine (PEI) transfection (Brunschwig, The Netherlands). 24 h after transfection cells were treated with 10 µg/ml BetA or 10 µM rapamycin for 18 h. HeLa cells were transiently transfected with tandem fluorescent p62 (pDest-mCherry-EGFP-p62, kindly provided by E Reits, AMC, The Netherlands), using PEI transfection. Twenty-four hours after transfection, cells were treated with 10 µg/ml BetA or 10 µM rapamycin for 12 h.

Degradation of long-lived proteins.
MCF-7 cells were seeded in 12-well plates. The next day cells were labeled with C-14 valine (0.2 µCi/ml). After 24 h, a cold chase using cell culture medium only was performed before adding BetA or rapamycin for various time points. Supernatants and cells were collected separately and both were precipitated for 30 min on ice in 10% TCA (trichloroacetic acid). Precipitates were collected via centrifugation for 10 min at 10 000 r.p.m. and subsequently dissolved in 0.5M NaOH. Radioactivity in supernatant and cell samples was measured and the ratio determined. The relative degradation ratio in the control cells was set to ‘one’ and compared with the degradation ratios in BetA and rapamycin-treated samples.

Western blot analysis.
Cells were lysed in 1x RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing complete proteinase inhibitor (Roche, Penzberg, Germany) and subjected to protein quantification using a BCA kit (Pierce, Thermo Fisher Scientific, Waltham, MA, USA). A quantity of 25 µg protein per lane was applied for SDS-PAGE. Subsequent blotting was performed using a PVDF membrane (Amersham Biosciences, Amersham, UK). Membranes were blocked in 5% milk (blocking buffer) in TRIS phosphate-buffered saline solution containing TWEEN 20 (0.2%) (TBST-T) for 1 h at room temperature. Primary antibody incubations were performed in 1% milk/TBS-T (LC3 antibody, tubulin antibody) or 5% BSA/TBS-T (ATG5 antibody, p62 antibody and ERK1/2 antibody) overnight at 4°C. Membranes were washed three times and incubated in blocking buffer for 1 h with a secondary HRP (horseradish peroxidase)-labeled antibody (anti-rabbit IgG (H+L) or anti-mouse IgG (H+L); Southern Biotech, Birmingham, AL, USA). For chemiluminescent visualization, Lumi-Light plus substrate (Roche, Penzberg, Germany) was used. Blots were analyzed by ImageQuant LAS4000. Band intensities were quantified using Image J software (NIH, Bethesda, MD, USA).

Statistical analysis.
Statistical analyses were performed with Prism 5 (GraphPad Software, La Jolla, CA, USA) applying Student’s t-test. Differences were considered significant with P-values <0.05 (*), <0.01 (**) and <0.001 (***)

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Conflict of Interest
The authors declare no conflict of interest.

Acknowledgements
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Supplementary figures

Supplementary Figure 1. BetA induces cell death in a concentration dependent fashion. HeLa cells were treated for 48 hours with indicated concentrations of BetA after which cell death was assessed by PI exclusion.

Supplementary Figure 2. Necrostatin blocks TNF-induced necroptosis. U937 cells were pre-treated with or without 10 μM zVAD.fmk and with or without 25 μM necrostatin-1 for one hour and subjected to 40 ng/ml TNF-α for 48 h after which cell death was assessed through PI exclusion. Mean ± S.D. of three independent experiments are shown, ***P<0.001.
Supplementary Figure 3. BetA induces autophagy and autophagic flux. HeLa cells were treated with (a) indicated concentrations of BetA for 18 hours or (b) indicated time points. LC3 processing was assessed via immunoblotting. Results for endogenous LC3 are shown. LC3 I (18 kDa) and LC3 II (16 kDa). Tubulin is used as a control. Band intensity of western blot was quantified using Image J software. Mean ± S.D. of three independent experiments are shown, *P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant. (c) HeLa cells were pre-treated for 1 hour with 10 µg/ml cycloheximide followed by 48 h of BetA treatment. P62 levels were determined via western blot. ERK1/2 is used as a control. Band intensity was quantified using Image J software. Single experiment is shown. (d) HeLa cells and A549 cells with tandem fluorescent P62 (pDest-mCherry-EGFP-p62) were treated with 10 µg/ml BetA or 10 µM rapamycin and after 18 h cells were analyzed by confocal microscopy. Representative pictures are shown of three experiments.
Betulinic acid induces a rescue autophagy response

Supplementary Figure 4. ATG deficiency results in increased BetA-induced cell death (a) Knockdown of ATG5 in HeLa, HeLa\LC3-GFP and MCF-7\LC3-GFP cells with shRNA control, shRNA ATG5 #1 and shRNA ATG5 #3 was assessed via immunoblotting. (b) MCF-7\LC3-GFP cells with ATG5 knockdown #1 and #2 and control cells were subjected to different concentrations of BetA. After 48 h after cell death was measured via PI exclusion and DNA fragmentation was assessed. Mean ± S.D. of three independent experiments are shown. (c) Mouse embryonic fibroblasts lacking either Atg5 or Atg7 and their respective control cells were subjected to different concentrations of BetA for 18 h and phosphatidylserine (PS) exposure was measured. Mean ± S.D. of three independent experiments are shown.
Chapter 3

Betulinic acid induces a novel cell death pathway that depends on cardiolipin modification

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“The more there is of mine, the less there is of yours”

Lewis Carroll, Alice in Wonderland
Abstract

Cancer is associated with strong changes in lipid metabolism. For instance, normal cells take up fatty acids (FAs) from the circulation, while tumour cells generate their own and become dependent on de novo FA synthesis, which could provide a vulnerability to target tumour cells. Betulinic acid (BetA) is a natural compound that selectively kills tumour cells through an ill-defined mechanism that is independent of BAX and BAK, but depends on mitochondrial permeability transition-pore opening. Here we unravel this pathway and show that BetA inhibits the activity of stearoyl-CoA-desaturase (SCD-1). This enzyme is overexpressed in tumour cells and critically important for cells that utilize de novo FA synthesis as it converts newly synthesized saturated FAs to unsaturated FAs. Intriguingly, we find that inhibition of SCD-1 by BetA or, alternatively, with a specific SCD-1 inhibitor directly and rapidly impacts on the saturation level of cardiolipin (CL), a mitochondrial lipid that has important structural and metabolic functions and at the same time regulates mitochondria-dependent cell death. As a result of the enhanced CL saturation mitochondria of cancer cells, but not normal cells that do not depend on de novo FA synthesis, undergo ultrastructural changes, release cytochrome c and quickly induce cell death. Importantly, addition of unsaturated FAs circumvented the need for SCD-1 activity and thereby prevented BetA-induced CL saturation and subsequent cytotoxicity, supporting the importance of this novel pathway in the cytotoxicity induced by BetA.
Introduction

Cancer is characterized by cell growth and proliferation, which requires an enormous surge in novel building blocks, such as nucleic acids, lipids and amino acids. To meet these requirements, cancer cells undergo major changes in their metabolism. For instance, lipid metabolism undergoes a dramatic shift towards lipid synthesis.1–3 For cellular lipid production, fatty acids (FAs) are needed as building blocks, which are either derived from exogenous sources or from de novo FA synthesis. Whereas normal cells prefer exogenous sources, tumour cells favour de novo synthesis.4–6 This differential usage of FA source is supported by observations that enzymes involved in FA synthesis, such as ATP-citrate lyase and FA synthase, are required for transformation of cells and upregulated in several tumour types.7–10 The main products of FA synthesis are palmitic acid (PA) and stearic acid, saturated FAs that are toxic for the cell at high levels and therefore converted to their monounsaturated forms (palmitoleic acid (POA) and oleic acid) by stearoyl-CoA-desaturase (SCD-1). This enzyme, located in the endoplasmatic reticulum, catalyses the introduction of a double bond at the ω-9 position of saturated FAs and is overexpressed in cancer cells.11–16 Unsaturated FAs are abundantly present in mammalian cells, as their structural properties are needed to maintain optimal fluidity of cellular membranes. Due to its tumour-selective activation, de novo FA synthesis has been suggested as an attractive target of anticancer therapy.8

Betulic acid (BetA) is a cytotoxic plant-derived compound that is tumour selective and does not kill normal cells.17–19 It induces apoptosis in a wide variety of tumour types through a not completely understood, but mitochondria-dependent mechanism.20–23 Importantly, this involves mitochondrial permeability transition (PT)-pore opening, but is independent of BAX and BAK, pointing to a distinct mechanism as compared with classical mitochondrial-dependent apoptosis. The general anti-cancer applicability of BetA and its selectivity suggests that it targets an essential and common pathway in tumour cells. Here we show that BetA affects mitochondrial morphology by changing the FA composition of cardiolipin (CL). CL is a unique mitochondrial phospholipid that contains four FA side chains. It is associated with several mitochondrial proteins and respiratory chain complexes and is essential for mitochondrial function and structural integrity of the organelle.24–27 In addition, CL is known to have an important role in apoptosis due to its interaction with core apoptotic machinery components, such as cytochrome c, caspase-8, BCL-2 family members and PT-pore components.25, 28–31

Our study demonstrates that BetA treatment increases the incorporation of saturated FAs in CL through inhibition of SCD-1. This is detrimental for mitochondrial morphology and leads to apoptotic cancer cell death.
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Results

Mitochondrial morphology is changed and CL saturation increased upon BetA treatment

In untreated cancer cells and normal cells, mitochondria appeared as a tubular network evenly divided throughout the cell (Figure 1a and Supplementary Figure 1). In contrast, in cancer cells treated with BetA the mitochondrial network changed in a concentration dependent manner and became more fragmented (Figure 1a and Supplementary Figure 1). Strikingly, this fragmentation is not observed in non-transformed human fibroblasts (Co18; Supplementary Figure 1). To gain more insight into these mitochondrial changes at the ultrastructural level, transmission electron microscopy was employed. Untreated cells contained mitochondria with a typical longitudinal cristae. Upon BetA treatment, mitochondria were more electron dense and displayed significant ultrastructural changes, in particular, concentric (circular shaped) cristae were evident (Figure 1b). Interestingly, such cristae abnormalities have previously been observed in tissues of Barth syndrome (BTHS) patients,32 a disease that is caused by a germline mutation in the Tafazzin gene, which encodes a transacylase involved in CL remodelling and maturation. Cells of BTHS patients display characteristic changes in CL, which cause mitochondrial ultrastructural as well as functional abnormalities.33

To determine whether the observed BetA-induced mitochondrial changes were also due to changes in the composition of CL, analysis of CL in several cancer cell lines was performed using high-performance liquid chromatography–mass spectrometry. A large variety of CL molecules is normally detected, which is due to a difference in the length and saturation of the four FA side chains in CL. In untreated tumour cells, CL appeared as six major clusters containing FA side chains of increasing length (from C64 to C74). The individual clusters are then further subdivided based on the level of saturation of their FA side chains (indicated by CL(64:X) to CL(74:X), where X denotes the number of unsaturations, that is, double bonds in the FA side chains; Figure 2a). The major CL cluster detected contained 68 side chain carbons (C68) and one to five double bonds (C68:1 to C68:5; Figure 2a and c). Importantly, BetA treatment of several cancer cell lines resulted in significant changes in the composition of CL that is evident from a shift in CL clusters to higher m/z values and hence less unsaturated CL species (Figure 2b). Zooming in on the major C68 cluster in these cell lines revealed a strong decrease in CL species with four or five double bonds and a concomitant increase of more saturated C68 CL species with only two or three unsaturations (Figure 2c and e, Supplementary Figure 2A, B and Supplementary Figure 6B and C). This profile is very similar to the one observed in fibroblasts from BTHS patients (Figure 2f and h), which as a consequence have major defects in their mitochondria, suggesting that the BetA-induced CL modification is instrumental in the mitochondrial changes.
The changes in FA composition of CL can be brought about by either de novo synthesis of complete CL molecules or by direct modification of the FA side chains by specific remodelling enzymes.\textsuperscript{34} To monitor the effect of BetA on de novo FA incorporation in CL, cells were incubated with heptadecanoic acid (C17:0), which is a low-abundant FA in normal physiology. Within a day, C17-containing CL clusters (odd numbered CL species) made up a significant proportion of the CL present in cells, indicating that this odd-chain FA was rapidly incorporated into CL (Figure 2i and k). These observations indicate that CL is relatively rapidly metabolized in cancer cells. In addition, the degree of unsaturation of C17-containing CL clusters was comparable to the existing (even numbered) clusters (Figure 2m), which suggested that exogenous C17:0 was taken up into the FA pool and also converted to its monounsaturated form, C17:1. Importantly, in BetA-treated cells rapid CL side chain turnover was also observed. However, in the presence of BetA the C17-containing clusters were more saturated (Figure 2j and l), a shift that was similar to the even-numbered counterparts (Figure 2e and n). This indicated that BetA-induced CL modification is a direct result from incorporation of novel FA side chains that in the presence of BetA are more saturated. Importantly, phosphaditylglycerol, which is the precursor of CL, also accumulated in a relatively saturated form in BetA-treated cells (Supplementary Figure 3A and B). Modification of CL was therefore dependent on a step further upstream in the biosynthesis of CL.

**SCD-1 activity is instrumental in BetA-induced mitochondrial damage**

In cells, de novo synthesized and exogenous saturated FAs are converted to unsaturated FAs via the enzyme SCD-1 (Figure 3a). In cancer cells SCD-1 RNA levels are higher as compared with normal fibroblasts (Supplementary Figure 4A). This is consistent with literature reports and suggests that cancer cells are more dependent on desaturase activity\textsuperscript{13,16} due to the activation of de novo FA synthesis in favour of FA uptake. Accumulation of saturated FAs in phosphaditylglycerol and CL could therefore be a direct result of dysfunctional FA conversion by SCD-1. To measure SCD-1 activity, stable isotope labelled $^2$H$^3$-C16:0 and $^2$H$^5$-C18:0 were added to live cells and conversion rates to unsaturated FAs were measured. Untreated cancer cells displayed a rapid conversion to the monounsaturated FAs, which was blocked by a specific SCD-1 inhibitor or genetic knockdown of SCD-1, confirming that SCD-1 was responsible for this conversion (Figure 3b and d and Supplementary Figure 4B and C). In line with the RNA levels, the SCD-1 activity is significantly lower in Co18 as compared with cancer cells.

Importantly, generation of unsaturated FAs by SCD-1 was strongly reduced by addition of BetA in all lines tested. This indicates that BetA inhibited SCD-1 activity (Figure 3b and c and Supplementary Figure 4C) and directly affects the pool of intracellular FAs, which then impacts on the saturation level of CL. In agreement, inhibition of SCD-1 with a commercial inhibitor also resulted in a rapid change in the composition of CL (Figure 3g) as well as in a
significant change in the size, ultrastructure and network of mitochondria (Figure 3h and Supplementary Figure 5). These data suggest that blocking FA desaturation is detrimental for the mitochondria through a rapid modification of CL side chains.

Figure 1. BetA-induced mitochondrial morphology changes (a) HeLa cells with MitoRFP were treated with vehicle (DMSO), 5 µg/ml or 10 µg/ml BetA and after 18h cells were analysed by confocal microscopy. (b) HeLa cells were treated with vehicle (DMSO) or 10 µg/ml BetA for 18 h after which cells were fixed and processed for TEM analysis. Pictures representative for 3 experiments are shown.

Unsaturated FAs revert CL modification and mitochondrial changes

High levels of saturated FAs are known to be cytotoxic and our current data point to a direct impact on the mitochondria.\textsuperscript{35,36} If this model is correct then addition of a surplus of saturated FAs would aggravate, while addition of unsaturated FAs would rather revert BetA-induced mitochondrial damage. Cells were therefore first treated with either PA (C16:0) or POA (C16:1). Addition of PA had relatively little impact on the composition of CL, as exemplified by the C68 cluster (Figure 3e and f and Supplementary Figure 6A and C), indicating that the added FA did not change normal CL metabolism. Single addition of POA slightly enhanced the level of unsaturation in CL as compared with untreated tumour cells (Figure 3e and f and Supplementary Figure 6A and C). However, the most striking effect was
observed when POA was added together with BetA as this prevented the induction of CL saturation significantly (Figure 3e and f and Supplementary Figure 6A and C). Similar effects were observed when CL saturation was induced by SCD-1 inhibitor (Figure 3g and Supplementary Figure 6D), indicating that the desaturase inhibition indeed directly impacts on CL due to a decrease in the production of unsaturated FAs. In contrast, PA addition rather enhanced the effect that BetA and SCD-1 inhibition exerted on CL saturation (Figure 3e and g and Supplementary Figure 6A and D). This confirmed that blocking SCD-1 activity is instrumental in BetA-induced CL changes as saturation can be prevented by the addition of unsaturated FAs, which bypasses the need for SCD-1 activity. The causal link between SCD-1 activity and CL saturation prompted us to investigate whether BetA-induced mitochondrial morphology changes were a direct result of CL saturation or whether they are induced through alternative pathways. Addition of either saturated (PA) or unsaturated (POA) FAs had no effect on the tubular mitochondrial network and cristae structure of mitochondria (Supplementary Figure 7A and B). However, the BetA-induced mitochondrial changes were clearly potentiated by addition of saturated FAs (PA) and more importantly prevented by unsaturated FAs (POA; Figure 3i and j), establishing a causal relationship between SCD-1 activity, CL FA composition and mitochondrial morphology.

**CL modification evokes cell death and decreases clonogenic survival**

Previously, we provided evidence that BetA induces mitochondrial leakage, cytochrome c release in a PT-pore dependent fashion and subsequent cell death.\(^{23}\) Cytochrome c release was indeed observed upon BetA treatment and with SCD-1 inhibitor treatment (Figure 4a and c), but interestingly, this release was significantly reduced when treatments were combined with POA. Vice versa, addition of PA enhanced the amount of cells showing cytochrome c release (Figure 4a and c), indicating that the inhibitory effects of BetA on SCD-1 activity directly impact on mitochondrial damage and lead to cytochrome c release. Moreover, these observations extend to the actual induction of cell death as measured by propidium iodide exclusion (Figure 4d and e). The validity of this novel cell death pathway was further substantiated with the use of SCD-1 inhibitor or genetic knockdown, which, next to mitochondrial defects, also induced cell death that could be blocked by exogenous addition of POA (Figure 4f and Supplementary Figure 8A and B). These data suggest that BetA-induced cell death by inhibiting SCD-1. It is important to realize though that BetA-induced death is more rapid than SCD-1-induced cell death, whereas the effects on SCD-1 activity are if anything more effective with SCD-1 inhibitors. This suggests that BetA may have additional effects that enhance the dependency on SCD-1 activity. In agreement, combining BetA with SCD-1 inhibitor resulted in enhanced cell death as compared with SCD-1 inhibitor alone, confirming that BetA has a stronger impact on cells. We hypothesize that BetA enhances the need for unsaturated FA by increasing the turnover of lipids and as
BetA-induced cell death by cardiolipin saturation

Figure 2. BetA-induced cardiolipin saturation (a) HeLa cells were treated for 18 h with vehicle (DMSO) followed by CL analysis by HPLC mass spectrometry. (b) HeLa cells were treated for 18 h with 10 µg/ml BetA followed by CL analysis by HPLC mass spectrometry. (c) Zoom in of CL C68 cluster (containing 2 x C16 and 2 x C18 fatty acids) of DMSO treated cells 18 h (d) Zoom in of CL C68 cluster of 10 µg/ml BetA treated cells. (e) Quantification of fold change of C68:X species in BetA treated (18 h) HeLa cells (n=8) compared to vehicle treated HeLa cells (n=8). (f) Zoom in of CL C68 cluster of control fibroblasts. (g) Zoom in of CL C68 cluster of BTHS patients. (h) Quantification of fold change of C68:X species in BTHS patients (n=3) compared to controls (n=3). HeLa cells were pre-treated for 4 hours with vehicle (DMSO) (i, j) or 50 µM heptadecanoic acid (C17:0) (k, l) after which vehicle (DMSO) i, k) or 10 µg/ml BetA (j, l) was applied for 18h. Zoom in of CL clusters of C68 and C69 (containing C17) are shown. (m) Quantitative analysis of CL content in C68 and C69 CL cluster of DMSO C17:0 treated cells (n=2). (n) Quantification of change of C68:X species in BetA treated HeLa cells compared to vehicle (DMSO) treated HeLa cells (n=2)
Figure 3. Desaturase activity is instrumental in BetA-induced mitochondrial damage (a) SCD-1 enzyme, introduces a double bond in saturated fatty acids at the n-9 position producing unsaturated fatty acids. (b) HeLa cells were treated with vehicle (DMSO), 150 nM SCD-1 inhibitor or 10 µg/ml BetA for 18 h after which \textsuperscript{2}H\textsubscript{2}-C16:0 was applied for additional 6 h. Conversion to \textsuperscript{2}H\textsubscript{1}-C16:1 was measured. Mean ± SD of 3 experiments are shown. ***P<0.001. (c) HeLa cells were treated with vehicle, 150 nm SCD-1 inhibitor or 10 µg/ml BetA for 18 h after which \textsuperscript{3}H\textsubscript{2}-C18:0 was applied for additional 6 h. Conversion to \textsuperscript{3}H\textsubscript{1}-C18:1 was measured. Mean ± SD of 3 experiments are shown. ***P<0.001. (d) Zoom in of CL C68 clusters. HeLa cells were incubated with vehicle (DMSO), 10 µg/ml BetA, 50 µM POA, 50 µM PA or a combination of 10 µg/ml BetA with 50 µM POA or 50 µM PA for 18 h after which CL analysis was performed using HPLC mass spectrometry (e) Quantification of CL saturation as measured in (d). Relative C68 CL saturation is shown which is calculated as (C68:2 + C68:3) / (C68:4 + C68:5). Mean ± SD of 2 experiments are shown. * P< 0.05; ns, not significant. (f) HeLa cells were subjected to vehicle (DMSO) or 200 nM SCD-1 inhibitor with or without 50 µM POA or 50 µM PA for 40 h after which CLs were analysed by HPLC mass spectrometry. Relative C68 CL saturation is shown which was calculated as (C68:2 + C68:3) / (C68:4 + C68:5). Mean ± SD of 3 experiments are shown. **P< 0.01; ***P<0.001. (g) HeLa cells were treated with vehicle (DMSO) or 200 nM SCD-1 inhibitor for 40h after which cells were analysed via TEM. (h) HeLa cells with MitorRFP were subjected to vehicle (DMSO) or 10 µg/ml BetA with or without 50 µM POA or 50 µM PA and after 18 h cells were analysed via confocal microscopy. Pictures representative for 3 experiments are shown. (i) HeLa cells were subjected to vehicle (DMSO), 10 µg/ml BetA with or without 50 µM POA or 50 µM PA and after 18 h cells were analysed via TEM. Pictures representative for 3 experiments are shown.
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such increases the incorporation of toxic saturated FA into lipids. Sustaining a stable pool of unsaturated FAs, by exogenous addition, therefore maintains the unsaturation grade of CL, mitochondrial integrity and thereby allows for cell survival. The maintenance of the unsaturated FA pool exerted long-term protection as it even resulted in the recurrence of clonogenic potential of BetA or SCD-1 inhibitor-treated cells. Single addition of these compounds resulted in a complete loss of clonogenic growth (Figure 4g and h), which was regained by the simple addition of POA. This effect was selective for this pathway and for instance not observed when cells were treated with classical chemotherapy such as etoposide (Supplementary Figure 8D). Taken together these data indicate that tumour cells carry an intrinsic mitochondrial vulnerability that can be effectively targeted by inhibition of SCD-1, using either specific inhibitors or the natural compound BetA and is not observed in normal fibroblasts. This vulnerability depends on enhancing the saturation of CL leading to altered mitochondrial structure, membrane permeability and as a consequence cell death and loss of clonogenic growth occur.

Discussion

Altered lipid metabolism is a key feature of tumour cells and required to sustain the constant need for new biomass. Our current study provides strong evidence for an unexpected mitochondrial vulnerability of tumour cells that depends on this de novo lipid synthesis. De novo FA synthesis generates intrinsically toxic FAs, which need to be converted by SCD-1 into monounsaturated FAs. Here we show that inhibition of this final step in the generation of preferred FAs results in cell death and loss of clonogenic growth. Interestingly, we confirmed that normal cells do not contain high levels of SCD-1 expression or activity consistent with the idea that these cells do not utilize de novo FA synthesis but instead acquire unsaturated FA from the surrounding. Although this effect of SCD-1 inhibition on tumour cells has been documented before, our data now provides an unexpected mechanistic explanation of this observed toxicity. Firstly, we show that SCD-1 inhibition quickly impacts on the composition of CL FA side chains, resulting in mitochondrial abnormalities, cytochrome c release and subsequent cell death. Secondly, we demonstrate that BetA, which is a potent anti-cancer agent, does so by inhibiting SCD-1 and therefore utilizes this tumour cell vulnerability. Together these data point to an upstream effect of BetA on CL biosynthesis. The fact that SCD-1 inhibitors mimic the effect of BetA and the observation that both can be reverted by the simple addition of unsaturated FAs all point to SCD-1 as the vulnerability in the CL biosynthesis pathway of tumour cells. Previously, we observed that BetA-induced cell death is tumour selective and that normal cells are not affected at doses that kill tumour cells both in vitro and in vivo. In agreement, the BetA or SCD-1-induced mitochondrial fragmentation in cancer cells is not detected in normal cells. The here proposed mechanism of action could
BetA-induced cell death by cardiolipin saturation

**Figure 4.** CL modification evokes cell death and decreases clonogenic survival. (a) HeLa cells were pre-treated for 24h with 50 µM POA after which vehicle (DMSO) or 10 µg/ml BetA was applied for 40 h. Other samples were left untreated for 24 h after which 50 µM PA with or without 10 µg/ml BetA for 40 h was applied. After treatment cytochrome c release was assessed via FACS. Data of 3 independent experiments are shown. * P< 0.05, ns not significant. (b, c) HeLa cells were pre-treated with 50 µM POA after which vehicle (DMSO) or 10 µg/ml BetA was applied for 48 h. Other samples were left untreated for 24 h after which 50 µM PA with or without 10 µg/ml BetA for 48h was applied. Cell death was assessed via PI exclusion. Mean ± SD of 3 experiments are shown. * P< 0.05, ns not significant. (d) HeLa cells were subjected to vehicle or 75 nM SCD-1 inhibitor with or without 50 µM POA or 50 µM PA for 72h after which cell death was assessed via PI exclusion. Mean ± SD of 3 experiments are shown. **P<0.01; ***P<0.001 (e) HeLa cells were treated for 48 h with vehicle (DMSO) or 10 µg/ml BetA with or without 50 µM POA or 50 µM PA after which fresh medium was applied for 72h. Cells were stained with crystal violet blue for 6h. Mean ± SD of 3 experiments are shown. **P<0.01. (f) HeLa cells were treated for 72 h with 75 nM SCD-1 inhibitor with or without 50 µM POA or 50 µM PA after which fresh medium was applied for 48h. Cells were stained with crystal violet blue for 6h. Mean ± SD of 2 experiments are shown. ***P<0.001.
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provide an explanation to this tumour selectivity. First, tumour cells appear addicted to de novo FA synthesis\textsuperscript{2–5} and as such continuously generate saturated FAs that need to be converted to unsaturated FAs. It is not completely clear why tumour cells prefer this synthetic pathway even in the presence of exogenous FAs. Except for adipocytes, this pathway is not utilized by normal cells, which acquire their FAs from exogenous sources.\textsuperscript{38} The latter provides a stable source of unsaturated FAs, and as such alleviates the need for desaturase activity in normal cells. Second, tumour cells have previously been shown to already contain CL species that are relatively more saturated as compared with normal cells.\textsuperscript{39} This could be a consequence of the apparently higher turnover of CL in tumour cells allowing for a less intense side chain modification or, alternatively, may be caused by the fact that mainly monounsaturated FAs are generated by de novo synthesis. Indeed, saturated FAs are abundantly found in cancer cells and also an increased content of monounsaturated at the expense of poly-unsaturated FAs is observed in transformed and cancer cells.\textsuperscript{40–42}

The higher saturation of CL suggests that tumour cells are closer to an unacceptable level of saturation than normal cells and therefore appear to be nearer to the tipping point of detrimental mitochondrial ultrastructural modifications and subsequent mitochondrial permeability. In agreement, BTHS patients contain a similarly low level of unsaturated FA side chains in CL and contain the same ultrastructural changes. However, this modification by itself is not sufficient for the actual mitochondrial leakage to occur as most cells in BTHS patients do not undergo cell death, suggesting that additional changes are needed. These could involve increased ROS production, which is often observed in tumour cells\textsuperscript{43,44} or a change in the way mitochondria are utilized in tumour cells, in particular in relation to the so-called Warburg effect.\textsuperscript{45,46} In agreement, our data show that BetA exerts cell death more rapidly and more vigorously than SCD-1 inhibition, which supports the idea that BetA is more potent in inducing cell death despite the fact that we observe more potent SCD-1 activity inhibition with SCD-1 inhibitors. We believe this is due to the fact that BetA enhances the need for new FA production. In essence we think that BetA not only stops the production of unsaturated FA, but on the other end increases the turnover of lipids and thereby the incorporation of saturated FA, which in the case of CL results in the toxic effects on mitochondria. This could explain the tumour selectivity as normal cells are not as dependent on SCD-1 and can maintain a relatively stable pool of unsaturated FA by uptake from the environment. The nature of this enhanced lipid turnover is not completely clear, but could for instance be a result of the massive induction of autophagy observed with BetA,\textsuperscript{67} which generates an enormous need for de novo membrane formation and thus lipid synthesis. Further insight into these additional changes may reveal another vulnerability that converges on the same pathway. Clues for these changes may come from neutrophils in BTHS patients as these display a relatively high level of apoptosis and indeed these patients frequently suffer from neutropenia.\textsuperscript{32,47}
The direct link between BetA-induced cell death and mitochondria is consistent with previous data which indicate that cell death ensues in a BAX and BAK-independent manner, but requires mitochondria and opening of the PT-pore. In agreement, cyclosporine A, an inhibitor of the PT-pore, blocks cell death induced by BetA and data not shown. Interestingly, recent data suggested that the F_o c-subunit ring of F_1F_o ATP synthase can form a channel, which can lead to uncontrolled depolarisation and MOMP when opened persistently. Whether this represents the PT-pore remains to be confirmed, but it is important to mention that F_1F_o ATP synthase as well as several of the other proposed constituents of the PT-pore associate with CL and CL can regulate PT-pore opening. Similarly, cytochrome c, which is released upon BetA treatment, interacts directly with CL through two independent binding sites. Moreover, Kagan et al. found that CL-bound cytochrome c acts as a peroxidase capable of catalysing H_2O_2-dependent peroxidation of CL and that this CL oxidation is an essential step in the release of cytochrome c during apoptosis. Whether the increased CL saturation has similar effects on the release of cytochrome c needs to be determined, but is a reasonable possibility. However, this initial release of cytochrome c from the inner membrane is not sufficient for apoptosis to occur. This requires mitochondrial outer membrane permeabilization either through BAX-dependent pore formation or through opening of the PT-pore. As BetA-induced death depends on the latter it seems likely to assume that CL saturation also affects the opening of the PT-pore.

In addition to the release of cytochrome c, mitochondrial fragmentation was rapidly observed in cells that have impaired SCD-1 activity and therefore modified CL FA side chains. The mitochondrial network is maintained by a continuous process of fusion (elongation of mitochondria) and fission (fragmentation of mitochondria). It is debated whether fusion/fission is important for cytochrome c release, but during apoptosis mitochondria are remodelled via activation of the fission machinery and synchronal neutralization of the fusion machinery. Importantly, this favoured mitochondrial fission occurs at the same time as BAX activation and permeabilization of the outer mitochondrial membrane, leading to cytochrome c release, suggesting that the events could be coupled. Mitochondrial fusion/fission itself is orchestrated by proteins such as Drp1 and Opa1, but also the structural, flexible, properties of CL. The observed mitochondrial fragmentation could therefore also be a direct effect of increased CL saturation, but how this would favour fragmentation is unclear and will require further analysis. Combined, our data point to a rapid effect of SCD-1 inhibition on the structural flexibility of CL and subsequently the mitochondrial integrity. Previous observations highlighted the importance of SCD-1 in tumours. Neoplastic cells contain higher levels of SCD-1 as compared with normal human skin fibroblasts and SCD-1 expression and activity are increased in several types of cancer. Moreover, normal cells appear to be resistant to the cytotoxic effect of SCD-1 inhibition. These observations suggest that a constantly active SCD-1 must be present in
cancer cells in order to maintain the level of unsaturated FAs to be incorporated in major cell lipids and thereby protect the tumour cells from excess saturated FAs. In agreement, knockdown of SCD-1 or inhibition with a specific small molecule inhibitor led to impaired biosynthesis of FAs, cholesteryl ester, triacylglycerol and phospholipid synthesis and induced death, confirming a pivotal role for SCD-1 in cancer. Several studies have reported the use of pharmacologic inhibitors of SCD-1 to reduce tumour growth in preclinical cancer models. In line with our data, toxicity induced by impaired SCD-1 expression sensitized cancer cells to the pro-apoptotic effects of PA. Our data now provide mechanistic insight and indicate that inhibition of SCD-1 rapidly changes the side chain saturation of CL and impairs normal mitochondrial structure. We conclude that CL is a crucial player in mitochondrial integrity that is strongly dependent on the level of saturation of its side chains. Targeting of this saturation or potentially even CL synthase in cancer cells provides a tumour-selective cell death mechanism that is induced by BetA.

**MATERIALS AND METHODS**

**Chemicals/antibodies**

BetA (BioSolution, Halle, Germany, >99% purity) was dissolved in dimethylsulfoxide at 4 mg/ml and stored at −80 °C. POA, PA, heptadecanoic acid and etoposide were purchased from Sigma-Aldrich (St Louis, MO, USA). SCD-1 inhibitor (cat# 1716) was from Biovision (Milpitas, CA, USA). Primary antibody to cytochrome c (6H2B4) was obtained from BD Pharmingen (Franklin Lakes, NJ, USA), secondary antibody fluorescein isothiocyanate conjugated antibody was obtained from Beckman Coulter (Fullerton, CA, USA). Crystal violet blue and glutaraldehyde were purchased from Merck (Darmstadt, Germany).

**Cell culture**

HeLa cells, A549 cells, MCF-7 cells, RKO cells and Co18 were obtained from the ATCC (Manassas, VA, USA). Cells were cultured in Iscove's modified Dulbecco's medium supplemented with 8% fetal calf serum, 2 mM L-glutamine, 40 U/ml penicillin and 40 μg/ml streptomycin.

**RNA interference**

Lentiviral vectors TRIPZ SCD small hairpin RNAs (GE Healthcare, cod. RHS4740-EG6319, Buckinghamshire, UK), or the non-silencing small hairpin RNA (GE Healthcare, cod. RHS4743) were transfected into HEK-293 T cells using polyethylenimine (Brunschwig, The Netherlands) according to vendor’s instructions. After 48 h and 72 h, the virus containing medium was collected and filtered using Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-100 membrane (Millipore, Billerica, MA, USA). The virus were resuspended in medium and HeLa cells were infected 24–48 h with virus containing medium supplemented with 10 μg/ml polybrene. Transduced cell populations were subsequently selected with 1
μg/ml puromycin and knockdown was induced by 72 h 2 μg/ml doxycycline treatment after which expression of the targeted proteins were determined via quantitative real-time PCR.

**RNA isolation and quantitative PCR**
RNA was isolated using the Superscript III Reverse Transcriptase Kit (Invitrogen, Waltham, MA, USA). Total RNA was retrotranscribed using Nucleospin RNA Kit (Macherey-Nagel, Düren, Germany). Quantitative PCR analysis was performed using the following primers: SCD-1-F (5'-TCTAG CTCTATACACACCA-3'), SCD-1-R (5'-TCGTCCTCAACTTATCTCCTCC-3'), GAPDH-F (5'-AACCCATCACCACCTTCA-3') and GAPDH-R (5'-TGGACTCC ACAGCTACTCA-3').

**Cell death analysis and cytochrome c release**
Cell death was determined by propidium iodide exclusion as described before.67 Cytochrome c release was performed according to the protocol of N. Waterhouse.68 Briefly, cells were permeabilized with 50 μg/ml digitonin/phosphate-buffered saline/KCl buffer, fixed for 30 min with paraformaldehyde and incubated in blocking buffer (3% BSA, 0.05% saponin, 0.02% azide and 1:200 normal goat serum in phosphate-buffered saline). Anti-cytochrome c incubation was performed overnight at 4 °C, after washing, fluorescein isothiocyanate conjugated secondary antibody was applied for 1 h at room temperature.

**Electron microscope**
After the indicated treatment, cells were fixed in 1% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (McDowell fixative) and postfixed with 1% osmiumtetroxide (OsO4, Electron microscopy sciences, Hatfield, PA, USA) and kaliumhexacyanoferrate (K3Fe(CN)6), VWR, Amsterdam, The Netherlands) in cacodylate buffer. Subsequently, the cells were dehydrated in an alcohol series and embedded into Epon (LX-112 resin Ladd Research, Williston, VT, USA). Ultrathin sections were collected on formvar-coated grids, counterstained with uranyl acetate and lead citrate and visualized with transmission electron microscope (FEI technai 12).

**Confocal fluorescence microscopy**
HeLa cells transiently transfected using polyethylenimine according to vendor’s instructions, with MitoRFP were grown on Poly-D-lysine coated 24 mm diameter glass cover slips in six-well plates. Cells were treated with BetA alone or in combination with the indicated concentrations of PA or POA for 18 h. A549, MCF-7, RKO and Co18 cells were grown on Poly-Dlysine coated 24 mm diameter glass cover slips in six-well plates. Cells were treated with vehicle or 10 μg/ml BetA for 18 h and stained 30 min with 100 nM mitotracker red CMXROS. The glass cover slips were mounted and placed in a Leica DMI 6000 (TCS SP8) microscope (with adaptive focus, Motorized XY-Stage and SuperZ Galvo) and a case
incubator at 37 °C for Z-stack measurements. Samples were analysed using Leica Las AF software (Leica Microsystems, Wetzlar, Germany).

**Mass spectrometry measurements of CL**
HeLa and A549 cells were harvested by trypsin and centrifugation at 300 g for 5 min at room temperature, washed once with phosphate-buffered saline, pelleted by centrifugation at 300 g for 5 min at room temperature. CL was analysed essentially as described previously.33 The relative abundances of the species in the sample-extracts were determined by high-performance liquid chromatography-mass spectrometry using a Surveyor high-performance liquid chromatography system hyphenated to a TSQ Quantum AM tandem mass spectrometer (Thermo Finnigan Corporation, San Jose, CA, USA). The MS was operated in the negative ion electrospray ionisation mode. Data were analysed using Xcalibur software (Thermo Fisher Scientific, Waltham, MA, USA).

**Desaturase activity measurements**
Cells were treated for 18 h with 10 μg/ml BetA or 150 nM SCD-1 inhibitor after which 50 μM 2H3-palmitate (2H3-C16:0; Cambridge Isotope Laboratories) or for HeLa cells, 50 μM 2H5-stearate (2H5-C18:0; Cambridge Isotope Laboratories) was added for an additional 6 h. Conversion of these saturated deuterium-containing FAs to unsaturated 2H3-C16:1 or 2H5-C18:1 was measured as described before.69,70

**Clonogenic growth**
HeLa cells were seeded in a 24-well plate and treated with 10 μg/ml BetA, indicated concentrations of SCD-1 inhibitor or with 1 μg/ml etoposide, with or without 50 μM POA or 50 μM PA for 48 h (BetA) or 72 h (SCD-1 inhibitor). After treatment, medium was changed and cells were left to grow for an additional 2 days. Cells were fixed in 6% glutaraldehyde and stained with crystal violet blue. Staining was dissolved in 0.5 ml 99% ethanol for 1 h with mild shaking. 100 μl of dissolved crystal violet staining was transferred to a 96-well plate and absorbance (590–630 nm) was measured (BioTek, Winooski, VT, USA).

**Statistical analysis**
Statistical analyses were performed with Prism 5 (GraphPad software, La Jolla, CA, USA) applying a 2-tailed unpaired Student t-test. Differences were considered significant when P-values, *P<0.05; **P<0.01; ***P<0.001.
BetA-induced cell death by cardiolipin saturation

Conflict of interest

The authors declare no conflict of interest.

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Supplementary figures

Supplementary Figure 1: BetA-induced mitochondrial morphology changes in cancer cells and not in normal cells. Indicated cell lines were treated with vehicle (DMSO) or 10 µg/ml BetA for 18 h after which cells were stained with 100 nM mitotracker red CMXROS and analysed by confocal microscopy.

Supplementary Figure 2: Induction of CL saturation upon BetA treatment. MCF-7 cells were subjected to vehicle (DMSO) or 10 µg/ml BetA for 24 h after which CL content was measured. Relative C68 CL saturation is shown, which is calculated as (C68:2 + C68:3) / C68:4. Mean ± SD of 2 experiments are shown. * P< 0.05; ns, not significant. (B) MCF-7 cells were subjected to vehicle (DMSO) or 10 µg/ml BetA for 24 h after which CL content was measured. Quantification of CL C68 cluster is shown. Mean ± SD of 2 experiments are shown.
BetA-induced cell death by cardiolipin saturation

Supplementary Figure 3: Heptadecanoic acid incorporates into CL. HeLa cells were pre-treated with vehicle (DMSO) or 50 µM heptadecanoic acid for 4 h after which vehicle (DMSO) (A) or 10 µg/ml BetA (B) was applied for 18 h followed by CL analysis by HPLC mass spectrometry. Note the higher abundance of the C17 containing PG (33:0) compared to that of PG (33:1) in BetA treated cells.

Supplementary Figure 4: Desaturase activity and levels in cancer cells. (A) Indicated cell lines were analysed for their relative SCD-1 mRNA levels by qPCR. (B) HeLa cells with SCD-1 shRNA were treated with doxycycline for 72 h to induce knockdown of SCD-1. Levels of SCD-1 mRNA levels were measured by qPCR. (C) Indicated cell lines were treated with vehicle (DMSO), 150 nM SCD-1 inhibitor or 10 µg/ml BetA for 18 h after which $^2$H$_3$-C16:0 was applied for additional 6 h. Conversion to $^3$H$_3$-C16:1 was measured. Mean ± SD of 3 experiments are shown. ***P<0.001, **P<0.01.
Supplementary Figure 5: SCD-1 inhibitor induced mitochondrial morphology changes. HeLa cells with MitoRFP were subjected to vehicle (DMSO), 200 nM SCD-1 inhibitor with or without 50 μM POA or 50 μM PA for 40 h after which cells were analysed by confocal microscopy. Representative pictures of 2 experiments are shown.
Supplementary Figure 6: CL levels of BetA and SCD-1 inhibitor treated cells (A) HeLa cells were subjected to vehicle (DMSO) or 10 µg/ml BetA with or without 50 µM POA or 50 µM PA for 18 h after which CL content was measured. Quantification of CL C68 cluster is shown. Mean ± SD of 5 experiments are shown (B) A549 cells were subjected to vehicle (DMSO) or 10 µg/ml BetA with or without 50 µM POA or 50 µM PA for 18 h after which CL content was measured. Relative C68 CL saturation of A549 cells is shown, which is calculated as (C68:2 + C68:3) / (C68:4 + C68:5). Mean ± SD of 3 experiments are shown. * P< 0.05; ns, not significant. (C) A549 cells were subjected to vehicle (DMSO) or 10 µg/ml BetA with or without 50 µM POA or 50 µM PA for 18 h after which CL content was measured. Quantification of CL C68 cluster is shown. Mean ± SD of 2 experiments are shown. (D) HeLa cells were subjected to vehicle (DMSO) or 200 nM SCD-1 inhibitor with or without 50 µM POA or 50 µM PA for 40 h after which CL content was measured. Quantification of the CL C68 cluster is shown. Mean ± SD of 3 experiments are shown.
Supplementary Figure 7: Effect of POA and PA on mitochondria (A) HeLa cells with MitoRFP were subjected to vehicle (DMSO), 50 µM POA or 50 µM PA for 18h after which cells were analysed via confocal microscopy. Pictures of 3 individual experiments are shown. (B) HeLa cells were treated with 50 µM POA or 50 µM PA for 18h after which cells were analysed via TEM.
Supplementary Figure 8: CL modification evokes cell death and decreases clonogenic survival (A) HeLa cells with induced knockdown of SCD-1 D6 and H12 were left for 5 days, after which cell death was assessed via PI exclusion. (B) HeLa cells were treated with indicated concentrations of SCD-1 inhibitor for 96 h after which cell death was assessed via PI exclusion. (C) HeLa cells were treated with vehicle (DMSO), 10 µg/ml BetA, 75nM SCD-1 inhibitor (+), 200 nM SCD-1 inhibitor (++) or combinations of BetA and SCD-1 inhibitor for 96h after which cell death was assessed via PI exclusion. (D) HeLa cells were treated for 48 h with vehicle (DMSO) or 1 µg/ml etoposide with or without 50 µM POA or 50 µM PA after which fresh medium was applied for 72 h. Cells were stained with crystal violet blue for 6 h. Mean ± SD of 3 experiments are shown.
Chapter 4

Betulinic acid induces a rapid form of cell death in colon cancer stem cells

Lisette Potze, Simone di Franco, Jan H. Kessler, Giorgio Stassi, Jan Paul Medema

Under revision at Stem Cell International

Door: Why it’s simply impassible!
Alice: Why? Don’t you mean impossible?
Door: No, I do mean impassible (chuckles) Nothing’s impossible!

Lewis Carroll, Alice in Wonderland
Chapter 4

Abstract

Cancer stem cells (CSCs) are considered to be the origin of cancer and it is suggested that they are resistant to chemotherapy. Current therapies fail to eradicate the CSCs and therefore select a resistant cell subset that is able to induce tumor recurrence. Betulinic acid (BetA) is a broad acting natural compound, shown to induce cell death via inhibition of the stearoyl-CoA-desaturase (SCD-1). This enzyme converts saturated fatty acids into unsaturated fatty acids and is over-expressed in tumor cells. Here we show that BetA induces rapid cell death in all colon CSCs tested and is able to affect the CSCs directly as shown via the loss of clonogenic capacity. Similar results were observed with inhibition of SCD-1, suggesting that SCD-1 activity is indeed a vulnerable link in colon CSCs and can be considered an ideal target for therapy in colon cancer.
BetA-induced cell death in colon cancer stem cells

Introduction

Colorectal cancer (CRC) is one of the major causes of death worldwide\(^1\) and the third leading cause of cancer death in the western world (WHO 2014, World Cancer Report 2014). Despite different and intensive treatments, current therapies are inadequate in eradicating the disease due to the occurrence of resistance. It has been suggested that such resistance depends on the intra-tumoral heterogeneity, in which the so-called cancer stem cells (CSCs) play a fundamental role.\(^2\) This cell subset is characterized by self-renewal and differentiation capacity\(^3, 4\) and it is regulated by a coordination of several pathways and signals responsible for the maintenance of such phenotype, as WNT\(^5-8\), BMP\(^9-12\) and Notch.\(^13-15\) CSCs presence was confirmed by several studies in different solid tumors, including colon cancer.\(^16-19\)

The resistance of colon CSCs to conventional therapy\(^17, 20-22\) makes these cells an obvious target to optimize current therapies. Strategies to identify and target specific cell surface markers are currently under investigation, with a particular focus on a functional characterization of the CSCs.\(^3, 23-26\) Despite more than two decades of research on the genetics of CRC\(^27\), it appears that we have only uncovered the tip of the iceberg.\(^28\) The extensive inter- and intra-tumor heterogeneity both genetically and therapeutically still forms an enormous challenge. We are only starting to understand the role and nature of genetic and epigenetic changes, the tumor microenvironment and the effects of different cell subsets within the tumor mass.\(^29-32\)

Current therapeutic approaches in colon cancer mainly utilize the proliferative capacity of tumor cells as a means to target the cancer. As cells can escape this type of therapy through multiple mechanisms, novel broad-acting compounds that target a distinct, but essential feature of the cancer cells are required. Such a therapy is able to target all the cells belonging to the tumor bulk, including the CSCs. A promising compound is represented by Betulinic Acid (BetA), which is a plant-derived compound that shows selective cytotoxicity for tumor cells sparing healthy cells.\(^33-35\) We have previously shown that BetA is very potent and effective in a wide range of tumor cells.\(^24, 34, 36, 37\) More importantly, we recently showed that BetA induces cell death via the inhibition of stearoyl-CoA desaturase (SCD-1) activity, an enzymatic activity that appears crucial to cancer but not normal cells.\(^38\) This enzyme converts saturated fatty acids derived from the \textit{de novo} fatty acid synthesis pathway into monounsaturated fatty acids and as such provides building blocks for new lipid biomass. The saturation grade of fatty acids in cells is crucial to maintain optimal fluidity of cellular membranes and it has been shown that inhibition of SCD-1 induces cell death in a variety of cells including lung CSCs.\(^39-41\) Due to its broad and potent activity we investigated the role of BetA in colon CSCs and also evaluated the effect of SCD-1 inhibition on these cells. Here we show that BetA and SCD-1 inhibition both induces cell death and loss of clonogenicity in colon CSCs. BetA induces a more rapid cell death in these cells.
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compared to SCD-1 inhibitor, thus suggesting that BetA is a very potent compound for cancer therapy.

Results

BetA induces a rapid cell death in colon cancer stem cells.

BetA has been shown to be very potent in killing several cancer cells after 48h with 60-90 % cell death in a p53 and BAX/BAK-independent manner\(^{36, 38}\) (see Table 1 for mutation analysis of colon CSCs). To determine whether BetA was also effective against colon CSCs, we used a panel of CSC cultures that were characterized for the most common stemness markers CD166, CD133\(^{16}\) and CD44v6\(^{3}\), and CK20, a colonic differentiation marker\(^{42}\) (Fig. 1A). We treated these spheroid cultures with BetA and observed a very rapid and potent induction of cell death. Within 2 h about 60 % of cell death was detected in BetA-treated GTG7 cells, while this resulted in 30 % and 40 % cell death in CC09 and Co147 cells respectively. Colon CSCs death induction is concentration- and time- dependent with cell death increasing to 60-80 % cell death after 8 h using the highest BetA concentration (Fig. 1B-G). Importantly, the rapid cell death is not dependent on culture conditions, but seemed to be selective for these spheroid cultured CSC cultures as normal colon cancer cell lines, such as RKO cells, only showed around 20% cell death at these early time points (Supplementary Fig. 1A-B). According with previous findings\(^{43}\), we observed BetA-induced cell death in both p53 mutant and p53 wild-type cell lines (Table 1).

Table 1. Mutation status of colon CSCs

<table>
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<th></th>
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Colon cancer stem cells lose their clonogenic capacity upon BetA treatment

One of the hallmarks of CSCs is represented by their resistance to therapy. Most conventional therapies induce cell death in differentiated tumor cells, but not in CSCs.\(^{44}\) As our spheroid cultures represent a mixture of CSCs and more differentiated tumor cells, we set out to directly analyze the effect of BetA on CSCs using WNT signaling activity as a read-out for stemness.\(^{5}\) Spheroid cells were therefore sorted for 10% low GFP, representing the more differentiated tumor cells, 10% high GFP, representing CSCs, and the total population. In all three cell populations BetA induced a rapid cell death suggesting that BetA targets both the more differentiated cancer cells as well as the CSCs (Figure 2A-D).
To confirm the cytotoxic effect on CSCs we looked into the functional impact of BetA treatment. One of the important features of CSCs is their ability to self-renew and to give arise to a heterogeneous population of CSCs and more differentiated cells. In other words, a single CSC is capable of forming a new colony with all cell types present.\textsuperscript{(15)} This capacity is present in the TOP-GFP high cells within the spheroids and defines the true CSCs. We therefore analyzed three distinct colon CSC cultures for their clonogenic capacity after treatment with different concentrations of BetA. BetA pre-treatment (2 h, single hit) clearly showed a strong reduction on stem cell frequency (60-80 % of reduction) in all the colon CSC cultures, in a dose-dependent manner (Figure 2E-G). In contrast, these short treatments did not affect the clonogenicity of RKO cells, confirming that BetA indeed targeted colon CSCs very effectively. (Supplementary Fig. 2) A separate measure of stemness is to determine the effect of therapy at later time points. We therefore analyzed the long-term effect of conventional chemotherapy (oxaliplatin) in comparison with BetA treatment on survival and regrowth of colon CSCs, using a CellTiter-Blue\textsuperscript{®} cell viability assay over a longer period. Treatment of two independent colon CSC cultures with oxaliplatin showed that despite an initial decrease in cell number, cultures eventually overcame the negative impact of chemotherapy and expanded over time, indicating that CSCs did not succumb to therapy even when higher doses of oxaliplatin were used (Figure 2H-I and Supplementary Fig. 3A-D). In contrast, treatment with increasing doses of BetA for only two hours revealed that long term expansion was completely annihilated at the highest concentrations of BetA (Figure 2H-I). Differential sensitivity was observed between the two CSC cultures, similar to the short-term toxicity assays with GTG7 being the most BetA sensitive cell line tested. In these cultures BetA concentrations of 5 µg/ml completely prevented outgrowth of cells, whereas in CC09, which were less sensitive to BetA, only the highest concentration of BetA (10 µg/ml) prevented outgrowth completely (Figure 2H-I and Supplementary Fig. 3 E-F). These data suggest that BetA affects colon CSCs viability and consequently long-term clonogenicity, while conventional therapy, such as oxaliplatin, gave rise to CSC-based resistance phenomena and subsequent recurrences in terms of growth.

**Inhibition of stearoyl CoA desaturase leads to cell death and loss of clonogenicity in colon CSCs**

We recently showed that BetA functions through inhibition of the enzymatic activity of SCD-1.\textsuperscript{(38)} In agreement, BetA treatment, SCD-1 inhibition or genetic knock down of SCD-1 in cancer cells, affect the saturation grade of fatty acids and specifically cardiolipin, resulting in impairment of the mitochondrial structure with consequent cell death.\textsuperscript{(38)} To validate whether BetA-induced death of colon CSCs is due to a similar SCD-1-dependent mechanism,
Figure 1. BetA-induced cell death in colon cancer stem cells. Flow cytometry analysis (A) of CD166, CD166, CD44v6 and CK20 in GTG7, CC09 and CO147 colon CSC lines (black histograms represent isotype control). GTG7 (B, C), CC09 (D, E) and CO147 (F, G) colon CSCs were treated for 2 h, 4 h or 8 h with indicated concentrations of BetA after which cell death was assessed via PI exclusion. In B, D, F representative FACS plots of GTG7, CC09 and CO147 colon CSCs are shown which were treated for 8 hours with DMSO or 10 µg/ml BetA. Mean ± SD of 3 experiments are shown. P < 0.05 (*); P < 0.01 (**); P < 0.001 (***)
cells were treated with a commercially available SCD-1 inhibitor. Interestingly, SCD-1 inhibition led to a time- and concentration-dependent cell death in both GTG7 and CC09. The timing of cell death was different in these cells, as GTG7 reaches a maximum cell death at 72 h, while this appears to take longer in CC09 (Figure 3A-B). In agreement with the effect observed with BetA treatment, SCD-1 inhibition dramatically reduced clonogenic potential of GTG7 and CC09 cells (Figure 3C-D). Henceforth, we conclude that BetA is an effective compound for the targeting of colon CSCs and this can be explained at least in part by the effect of BetA on SCD-1 activity in CSCs.

Discussion

CSCs are known to be more resistant to conventional treatment due to their (acquired) resistance mechanisms, such as high levels of ATP-binding cassette (ABC) transporters and anti-apoptotic molecules, active DNA-repair, in some cases reduced proliferation and the production of growth factors that confer refractoriness to anti-neoplastic treatments. The escape mechanisms from standard therapies were shown both in vitro and in vivo. In particular chemotherapy treatment of colon cancer results in an increase in CD133+ (oxaliplatin treatment), or ESA+/CD44+/CD166+ (irinotecan treatment) cell populations, suggesting that these markers identify the CSC population, which are more resistant than their differentiated counterpart. CSC resistance mechanisms are frequently suggested to provide an explanation for minimal residual disease in which an initial response is followed by recurrences due to survival and subsequent expansion of colon CSC. Here we show that BetA and SCD-1 inhibition can both circumvent this CSC resistance and induced cell death and loss of clonogenicity in colon CSCs. Our current study provides evidence for a vulnerability in colon CSCs in the SCD-1 pathway. Previously, we have also shown that this BetA-induced killing depends on the mitochondria, but occurs in a BCL-2 family-independent manner. This may be essential for its capacity to kill CSCs as we and others recently provided evidence that CSCs protect themselves from classical chemotherapy using BCLXL-dependent mechanisms. As BetA kills cells in a BCL-2 family independent manner it is expected to also target these CSCs. BetA has previously been shown to be tumor specific and not targeting healthy cells. No toxicity to BetA was observed in mice treated with BetA as well as vehicle liposomes, as both had the same amount of proliferating cells in the small intestine. On top of this, we showed that non-transformed fibroblasts are insensitive to BetA as well as SCD-1 inhibition. Cancer cells have reprogrammed their metabolism with increased glycolysis and lipogenesis (de novo fatty acid synthesis) to maintain the need for new biomaterials. This reprogramming needs to already be present in the tumor initiating cell, either pushing the cell fate toward transformation or creating an appropriate “metabolic state” required for
Chapter 4

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BetA-induced cell death in colon cancer stem cells

**Figure 2. BetA-induced loss of clonogenicity in colon cancer stem cells.** (A, B) GTG7 colon CSCs were sorted into three fractions, high GFP (indicating the stem cells), low GFP (indicating more differentiated cells) and total cells. Overnight adhered cells were treated with vehicle (DMSO) or 10 µg/ml BetA for 2 h, after which cell death was measured via PI exclusion. Representative FACS plots are shown in A. Mean ± SD of 3 experiments are shown. P < 0.001 (**). (C, D) CC09 colon CSCs were sorted into three fractions, high GFP (indicating the stem cells), low GFP (indicating more differentiated cells) and total cells. Overnight adhered cells were treated with vehicle (DMSO) or 10 µg/ml BetA for 2 h, after which cell death was measured via PI exclusion. Representative FACS plots are shown in C. Mean ± SD of 3 experiments are shown. P < 0.001 (**). Limiting dilutions experiments were performed and stem cell frequency was calculated in GTG7 (E), CC09 (F) and CO147 (G) colon CSCs that were pre-treated for 2 h with indicated concentrations BetA. Mean ± SD of 3 experiments are shown. P < 0.05 (*); P < 0.01 (**); P < 0.001 (**). GTG7 (H) and CC09 (I) colon CSCs were treated with indicated concentrations BetA for 2 h or with 0.5 µM oxaliplatin for 24 h after which cell numbers were measured at different time points using cell titer blue.

**Figure 3. SCD-1 inhibitor-induced cell death and loss of clonogenicity in colon cancer stem cells.** GTG7 (A) and CC09 (B) colon CSCs were treated for 24 h, 48 h, 72 h or 96 h with indicated concentrations of SCD-1 inhibitor after which cell death was assessed via PI exclusion. Mean ± SD of 3 experiments are shown. P < 0.05 (*); P < 0.01 (**) P < 0.001 (**). Limiting dilutions experiments were performed and stem cell frequency was calculated in GTG7 (C) and CC09 (D) colon CSCs that were pre-treated for 48 h (GTG7) or 72 h (CC09) with 1µM SCD-1 inhibitor. Mean ± SD of 3 experiments are shown. P < 0.001 (**).
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Tumorigenesis.\textsuperscript{48} Although there is no direct proof, a growing body of evidence suggests that metabolic reprogramming is at the core of cancer and it may provide a vulnerability of cancer cells.\textsuperscript{48-50} To maintain a pool of healthy fatty acids needed for new biomaterials, tumor cells start to depend on de novo fatty acid synthesis, while normal cells hardly utilize this pathway as they acquire unsaturated fatty acids from their surroundings. Newly generated fatty acids are fully saturated and are relatively toxic to the cells necessitating their conversion to unsaturated fatty acids by introduction of a double bond at the ω-9 position by the enzyme SCD-1.\textsuperscript{40, 51-54} This points out the need for SCD-1 activity in cancer cells, but could also hint to a vulnerability of cancer cells to this pathway. In several types of cancer, including colon cancer, SCD-1 expression and activity are increased \textsuperscript{38, 54, 55}, moreover SCD-1 levels were found elevated in \textit{in vivo} models that were genetically predisposed to develop certain cancers.\textsuperscript{56} These observations suggest that SCD-1 activity is needed in cancer cells in order to maintain the levels of unsaturated fatty acids and by doing so protecting tumor cells from a surplus of toxic saturated fatty acids.\textsuperscript{40, 55, 57} Indeed when inhibiting SCD-1 by small molecule inhibitors or genetic knockdown induction of cell death was observed in cancer cells\textsuperscript{38, 57} and this is a result of a strong switch in the saturation grade of the mitochondrial lipid cardiolipin and as a consequence the induction of mitochondrial fragmentation and induction of cell death. Here we show that colon CSCs are also sensitive to inhibition of the SCD-1 enzyme. The vulnerability to inhibition of SCD-1 activity was also recently observed in another study where inhibition of SCD-1 in lung CSCs resulted in a reduced stem cell marker expression and induced cell death.\textsuperscript{41} These data are in agreement with what we observed in our colon CSCs model. One striking observation is the velocity of cell death induction, which appears to be much higher as compared to classical colon cancer cell lines. This is specific for BetA and not observed with SCD-1 inhibition, which appears as effective in cancer cells and colon CSC cultures.\textsuperscript{38} We previously have hypothesized that the rapid effect of BetA is due to a combined effect of BetA, on one hand blocking the production of unsaturated fatty acids, but on the other hand increasing turnover of lipids and thereby the incorporation of saturated fatty acids.\textsuperscript{38} This concept therefore may point to an additional effect of BetA on colon CSC cultures that strongly enhances the impact on these cells. At this point we have no definitive proof as to this effect, but it is interesting to note that colon CSCs can be identified by their high level of lipid droplets when compared with differentiated tumor and even more so when compared to cancer cell lines.\textsuperscript{58} Lipid droplets are fat storing organelles, that are suggested to be involved in the storage, transport and metabolism of lipids, in signaling, and as a specialized microenviroment for metabolism.\textsuperscript{59, 60} SCD-1 plays an important role in lipid droplets formation and size. In SCD-1 WT C. elegans, large-sized lipid droplets were observed, while in SCD-1 mutant C. elegans small-sized lipid droplets are found.\textsuperscript{61} Small-sized lipid droplets are also found in patients with Berardinelli-Seip congenital lipodystrophy, which have decreased SCD-1 activity.\textsuperscript{62} These lipid droplets
BetA-induced cell death in colon cancer stem cells

appear to be crucial for the CSCs and we hypothesize that BetA might interfere with these lipid droplets and therefore induces a very rapid induction of mitochondrial damage.\(^{(38)}\)

We conclude that SCD-1 is a crucial player in the maintenance of stemness of cancer cells and inhibition is incompatible with long-term survival. Targeting the activity of this enzyme in CSCs provides an effective means to target these cells and can be achieved either with direct chemical inhibitors or even more effectively with BetA.

**Material & Methods**

**Chemicals/antibodies**
Betulinic acid (BioSolution Halle, Germany, >99% purity) was dissolved in DMSO at 4 mg/ml and stored at -80°C. Oxaliplatin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Stearoyl-CoA desaturase inhibitor (cat# 1716) was from Biovision (Milpitas, CA, USA).

**Isolation, Culture and Characterization of Colon Cancer Cells**
Colon cancer tissues were collected at the Department of Surgical and Oncological Sciences, and CEMM Department, in accordance with the ethical standards of the University of Palermo and AMC institutional committees. Colon spheroid cultures were derived from colorectal cancer patients and maintained in stem cell medium (advanced DMEM/F12 (Gibco) supplemented with N2 Supplement (Gibco), 6 mg/ml glucose, 5 mM HEPES, 2 mM L-glutamine, 4 µg/ml heparin, 50 ng/ml epidermal growth factor and 10 ng/ml basic fibroblast growth factor) as previously described \(^{(16)}\). The colon CSC lines obtained were subjected to genotypic characterization in order to validate each cell line’s individuality and their mutational status (Table 1), and further tested for their ability to generate tumor xenografts that replicated the parental histology. Colon CSCs were characterized by their expression of the putative cancer stem cell markers CD166-PE (3A6; mouse IgG1k; BD Biosciences), CD133-APC (293C3; mouse IgG2bk; Miltenyi) and CD44v6-APC (2F10; mouse IgG1; R&D systems), and CK20 (Ks20.8; mouse; IgG2ak), a colonic differentiation marker, data were acquired by flow cytometry using a BD ACCURI C6 and analyzed using the FlowJo software (Tree Star Inc., Ashland, OR, USA). CK20 was detected by using a goat anti-Mouse IgG (H+L) secondary antibody, Alexa Fluor®-488 conjugate. RKO cells were obtained from ATCC (Manassas, VA, USA) and maintained in IMDM supplemented with 8% FCS, 2 mM L-glutamine, 40 U/ml penicillin and 40 mg/ml streptomycin. GTG7 and CC09 cells bear the TCF Optimal Promoter (TOP)-green fluorescent protein (GFP) construct.\(^{(5)}\) These cells, derived from single-cell cloned TOPGFP cultures, still show a big heterogeneity in Wnt signaling levels, which positively correlates with cancer stemness in colon CSCs.\(^{(63)}\)

**Cell death analysis**
Cell death was determined by PI exclusion. In short, 50 000 single colon CSCs were seeded in a 24-well plate and allowed to attach overnight. Cells were treated for indicated time-
points with BetA or SCD-1 inhibitor and harvested. The cell pellet was resuspended in 200 μl medium and stained with PI at 0.5 μg/ml just before measuring by flow cytometry (FACSCanto, BD Biosciences). Samples were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

**Cell Proliferation Assay**
For determination of cell viability, 1000 cells per well were plated in repellent surface for low attachment 96-well plates (CELLSTAR® Cell-Repellent Surface, Greiner Bio- One) in medium containing vehicle (DMSO), 0.5 μM oxaliplatin or increasing doses of BetA. Viable cells were determined at indicated time-points using Cell Titer Blue (Promega, Fitchburg, WI, USA) by measurement of absorbance at 540 nm in a Synergy plate reader (Biotek). Cell viability at each concentration was expressed as OD cells – OD background.

**Limiting dilution assay**
The self-renewal capacity of colon CSCs was assayed by dissociation of colon cancer spheroids. Colon CSCs were cultured in adhesion o/n and pre-treated 2 h with indicated concentrations BetA or pre-treated 48 h or 72 h with SCD-1 inhibitor after which cells were collected and plated at serial dilution (1, 2, 4, 8, 16, 32, 64 and 128 cells/ well) in 96 well microplates with flat bottom and repellent surface for low attachment (CELLSTAR® Cell-Repellent Surface, Greiner Bio- One), using a FACS Aria II. Results were statistically evaluated after 3 weeks by using the Extreme Limiting Dilution Analysis (ELDA) software.\(^{64}\)

**Statistical analysis.**
Results are shown as the mean ± SD for at least three repeated independent experiments for each group. The mean and SD were obtained by analyzing three replicates using Prism 5 (GraphPad Software, La Jolla, CA, USA) applying a 2-tailed unpaired Student t-test. Differences were considered significant when p values P < 0.05 (*); P < 0.01 (**); P < 0.001 (***)

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Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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References


BetA-induced cell death in colon cancer stem cells


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BetA-induced cell death in colon cancer stem cells


Supplementary figures

**Supplementary Figure 1. BetA-induced cell death in colon cancer cells** (A, B) RKO cells were treated for 2 h, 4 h, and 8 h with indicated concentrations of BetA after which cell death was assessed via PI exclusion. Representative FACS plots of RKO cells treated for 8 h with DMSO or 10 µg/ml BetA are shown in A. Mean ± SD of 3 experiments are shown. P < 0.05 (*); P < 0.01 (**); P < 0.001 (***)

**Supplementary Figure 2. Clonogenicity in colon cancer cells** RKO cells were pre-treated for 2 h with indicated concentrations BetA after which limiting dilution experiments were performed and stem cell frequency was calculated.
BetA-induced cell death in colon cancer stem cells

**Supplementary Figure 3. Long term assay on CSCs.** (A) GTG7 and CC09 (B) colon CSCs were treated with 0.5 µM oxaliplatin for 24 h or indicated concentrations BetA (µg/ml) for 2 h after which cells were left to grow. Pictures (10x magnification) were taken at day 10 after which the number of cells were determined with cell titer blue. (C) GTG7 and CC09 (D) colon CSCs were treated with indicated concentrations oxaliplatin (µM) for 24 h after which cells were plated and cell numbers were measured at different time points using cell titer blue. (E) GTG7 and CC09 (F) colon CSCs were treated with indicated concentration oxaliplatin (µM) for 24 h after which cells were left to grow. Pictures (4x magnification) were taken at day 15 after which the number of cells were determined with cell titer blue.
Chapter 5

Improved identification of lipids using physico-chemical properties: application to lipidomics analysis of Betulinic acid treatment

Lisette Potze, Mia L. Pras- Raves, Henk van Lenthe, Martin A.T Vervaart, Jan H. Kessler, Angela C. Luyf, Jan Paul Medema, Antoine H. C. van Kampen, Frédéric M. Vaz

Under review at Journal of Lipid Research

“Who in the world am I? Ah, that’s the great puzzle.”

Lewis Carroll, Alice in Wonderland
Abstract

The correct identification of all metabolites in LC-MS lipidomic experiments is a time-consuming step in the data analysis process. Here we describe a method which not only uses the mass and retention time of peaks, but also incorporates the elution pattern of the phospholipid cluster to improve the identification of phospholipid species. Thus, even at low resolution (<10,000), a clear distinction can be made between chromatographically separated isobaric lipids such as phosphatidyglycerol and bis(monoacylglycerol)phosphate, between phosphatidylethanolamine (PE) and plasmalogen PE, and between phosphatidylserine and phosphatidylinositol. The method is illustrated using a lipidomics data set acquired on a low resolution triple quadrupole MS to investigate the effect of the anti-cancer drug Betulinic acid in HeLa cells.

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Introduction

Lipidomics is the comprehensive study of the lipid composition of cells, organelles or biofluids\(^\text{[1]}\). The combination of (ultra-)high-performance liquid chromatography ((U)HPLC) and mass spectrometry (MS) is instrumental in the measurements of lipids in complex samples\(^\text{[2]}\). Despite the progress and technical advances in the field in the last decade, there are still many challenges. First, the chromatographic separation of lipids is challenging due to the complexity of the samples, matrix effects, and the sheer number of different, but very similar, metabolites\(^\text{[3, 4]}\). Inadequate separation will produce complex mass spectra that are more difficult to analyze due to, for example, overlapping peaks in the chromatographic and/or mass domain. Secondly, current data pre-processing methods produce peak tables containing monoisotopic and isotope peaks based on their retention time and m/z values, often still requiring intervention and correction by experts experienced with LC-MS spectra to reduce the number of false positive and missing peaks. This makes the pre-processing of raw MS data very time consuming but, surprisingly, data pre-processing receives relatively little attention in literature\(^\text{[5]}\). Finally, the assignment of metabolites to all peaks in the peak lists is a major bottleneck. Frequently, only a small fraction of the observed peaks can be reliably assigned to a compound. This complicates further data analysis and comprehensive biological interpretation. The assignment of compounds generally requires a combination of knowledge of expected metabolites, internal standards, fragmentation patterns and spectral databases to which MS spectra can be compared\(^\text{[6]}\). There are several repositories and search engines to assist in the identification of phospholipids (PLs), including Lipid Maps (Lipidomics Gateway), containing over 30,000 lipid species\(^\text{[3, 7]}\) and Lipid Search\(^\text{[8]}\) consisting of more than 200,000 structures. The LipidBlast database combines computer-generated spectra and experimental fragmentation data, describing about 120,000 structures for lipids\(^\text{[9]}\) while online databases such as VaLID\(^\text{[5]}\) aid in the identification of glycerophospholipids based on their molecular mass and provide visualization of the molecules. Database searches, however, are generally conducted peak by peak, thus ignoring the pattern that is formed by the whole cluster of PLs that belong to the same class, a pattern which is caused by similarities and subtle differences in their physico-chemical properties. In a normal-phase LC system, PLs with the same head group will have roughly the same RT, but within such a cluster, the inclusion of an extra $-\mathrm{CH}_2-$ group or the presence of an additional double bond in one of the side chains will influence the observed RT of the PLs. The resolution of the MS measurements is another important factor in the ease of the identification. Higher resolutions yield more accurate m/z values, which enables the determination of the exact elemental composition of the underlying metabolite, even though this may not always pinpoint a unique identifier (for example, bis(monoacylglycerol)phosphate (BMP) and phosphatidylglycerol (PG) have the same elemental composition but different chemical structures). Querying m/z values from low-resolution MS (resolution < 10,000) in a reference database results in a long list of
candidate metabolites for every peak, rendering the identification process time-consuming and error-prone. Many clinical diagnostics laboratories currently make extensive use of low-resolution single- or tandem mass filter (quadrupole) setups\textsuperscript{(10)}, for which the usefulness of brute force database searches, without regard for the context of the peak, is limited.

With the aim of reducing the amount of work that is required for the analysis and interpretation of the results of a data set after pre-processing, we describe a method which not only uses the mass and retention time of peaks, but also incorporates the elution pattern of the phospholipid cluster to improve the identification of phospholipid species. We have integrated our identification method with XCMS\textsuperscript{(11)}, which is an R package widely used for the analysis of metabolomics data, into a pipeline which includes functionalities for isotope correction and normalization of intensities for assigned peaks (Figure 1).

To test our lipidomics pipeline we made use of a data set of HeLa cells treated with Betulinc acid (BetA). BetA is a plant-derived compound that shows selective cytotoxicity for tumor cells while healthy cells remain unaffected\textsuperscript{(12, 13)}. Recently, we showed that BetA induces apoptosis by inhibition of stearoyl-CoA desaturase 1 (SCD-1), resulting in changes of the side-chain saturation of cardiolipin (CL, a phospholipid mainly located in the inner membrane of mitochondria) and impaired mitochondrial morphology. We concluded that CL is a crucial player in mitochondrial integrity that is strongly dependent on the level of saturation of its side chains. However, in the above-mentioned study we only focused on PG, the precursor of CL, and on CL itself\textsuperscript{(13)}. Therefore, we revisited this data set to perform a complete lipidomics analysis to investigate the effect of BetA on the full lipidome and demonstrate the usefulness of our pattern recognition algorithm.

**Results**

**Data processing:**

We developed a method that facilitates the assignment of lipid compound names to peak groups originating from the same class of PLs. We have integrated our identification method with XCMS\textsuperscript{(11)}, a software package in the R programming language (http://www.R-project.org) which is widely used for the analysis of metabolomics data.

A typical full scan analysis yields between 1000 and 10,000 peak groups. Each of these peak groups is characterized by the median m/z and median RT of all the peaks from the different samples. For pre-processing packages such as XCMS\textsuperscript{(11)}, this is the final output. The assignment of compound labels to each peak group is then in the hands of the user.
Improved identification of lipids using physico-chemical properties

Figure 1: Schematic representation of the lipidomics pre-processing pipeline. Raw data files are obtained for every sample; the pre-processing pipeline using XCMS, followed by identification, isotope correction and normalization yields the final annotated peak list.

Plotting the peak groups (Figure 2) immediately allows the experienced eye to observe clusters, which are the result of groups of phospholipids with the same head group but with different side-chain compositions. The addition of internal standards (IS) per class of PLs to the samples not only serves for normalization and quantification purposes: identification of the IS peaks in the peak group list enables the determination of the location of the entire cluster of peaks originating from compounds from the same class, and aids in the identification of all peaks in this region. Specific head group scans, such as neutral-loss or precursor-ion scans, can also be helpful in locating clusters of PLs. Rather than relying only on the match to a theoretical mass of a compound, our method makes use of the systematic pattern that is formed by all the compounds in the same PL class upon normal-phase LC separation. This pattern is the result of differences in mobility on the LC column of PL molecules with different side-chain compositions. On a normal-phase column, PLs with longer side chains have a lower RT, due to a decreased polarity of the molecules. In addition, as the number of double bonds in the combined side chains increases, the RT decreases. These patterns can be used to aid in the assignment of PL names to peak groups. This is illustrated in Figure 3A and B, which shows the CL region in close-up, and described in the methods section in detail. Resulting data files are available as supplemental files (supplemental files 1-4).
Figure 2: All peaks in lipidomics data set. Clusters observed in PL peak groups in negative (lower panel) and positive scan mode (top panel, only precursor ion scan m/z 184 (PR184) is shown). Every peak group that was obtained using XCMS is plotted as a black dot. Several clusters of peak groups can be identified and are labeled in the figure, with a line showing the direction of the peak groups in each cluster. Peak groups observed in positive PR184 scan mode are shown above the horizontal line at RT=8.5 minutes; below the line are the peak groups in negative mode.
Improved identification of lipids using physico-chemical properties

Figure 3: Pattern observed in doubly charged cardiolipin. (A) The full CL cluster, starting in the upper left-hand corner with the IS peak, CL(56:0). (B) Close-up of CL(64:x) up to CL(66:y). For chromatographic separation with a normal-phase column, the RT of the compounds decreases with increasing (combined) chain length and number of double bonds in the chains. Dots represent the median location of all samples in a peak group. The size of the circle indicates the relative average intensity of those peaks on an arbitrary scale.
For the data set described in this paper, which was measured at relatively low resolution (approximately 2000 at m/z 500), we were able to identify metabolites from 9 classes in negative scan mode (BMP, CL, mICL, PG, PA, PE, pPE, PI, PS), and as many as 40% of all peak groups could be identified as either belonging to monoisotopic peaks (e.g. metabolites) or to corresponding isotope peaks. In positive scan mode (precursor-ion scan for m/z 184.1 (PR184) and neutral-loss scan for m/z 141.1 (NL141)), 10 classes (LPC, LpPC, PC, pPC, aPC, SM in PR184 and LPE, PE, pPE, aPE in NL141) and more than half of all peak groups could be identified in PR184 and NL141, (Table 1, supplemental tables I-IV). Other regions in the m/z versus RT space contain either noise, peaks from unknown PL classes, or peaks originating from compounds other than PLs. In neutral-loss or precursor-ion spectra, where only one class of PL is studied, the success rate of the identification is higher than for full-ion scans (supplemental tables II and III). This method works equally well for low-resolution lipidomics data as for high-resolution measurements (data not shown).

![Figure 4](image)

**Figure 4: Total phospholipid levels in BetA or vehicle treated cells.** Highly abundant PLs (PE, PS, PC and SM) (A) and less abundant PLs (BMP, CL, LPC, PG, PA) (B) for DMSO (vehicle) and BetA treated cells are depicted.

* = P<0.05, ** = P<0.001

**Analysis of the BetA data set using the pattern recognition algorithm**

Differences between the cells treated with vehicle (DMSO) and BetA treated cells were found in different phospholipid classes (Figure 4A and B). The most significant changes occurred in the total levels of BMP and PG: BetA reduces total BMP levels while PG levels are increased after treatment with BetA. There were effects on PC and LPC levels (elevated in BetA) as well, although they were less pronounced. Further analysis within the PL clusters showed that BetA induced an increase in intensity for saturated CL (number of double bonds less than 4) and a decrease for poly-unsaturated CL (4 double bonds and up), compared to the control (DMSO) samples (Figure 5). This effect is not observed in PG or BMP, even though PG is the precursor for CL (Supplemental Figure IA and IB).
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Figure 5: Heatmap of differences in cardiolipin composition. The cardiolipins have been sorted by total number of double bonds in the combined side chains; only CL(64:x) through CL(70:y) are shown. A clear distinction between mostly saturated CLs (number of double bonds < 4) and poly-unsaturated CLs (number of double bonds > 3) can be seen.
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Discussion

Being able to automatically identify metabolites greatly speeds up the data analysis process for metabolomics experiments. Querying m/z values in different (online) databases is not always practical: it often yields multiple assignments for a metabolite, sometimes none. In any case, the analysis of the data sets requires considerable attention and time from metabolomics experts which in practice results in a bottleneck since individual datasets contain hundreds to thousands of peak groups (and considerably more for high-resolution data sets). The pre-processing lipidomics pipeline described in this paper is suitable both for low-resolution (the data set described here has a resolution of approximately 2000 at m/z 500) and for high-resolution data (120,000 at m/z 500 for a Thermo Scientific Q-Exactive plus). For low-resolution data, the greatest benefit of our method is in the disambiguation of peaks with almost identical mass, such as PE and pPE. For high-resolution data, the main advantage is that a great number of peaks can be rapidly and reliably identified.

The pipeline yields peak tables and plots per class and per section of a class for all identified PLs, such as illustrated for CL in Figure 3. This enables the user to check whether the identification process has given the correct result. In many cases, analyzing the lipidome of a biological system gives considerably more insight into the underlying biochemical processes than one would get when focusing on only one PL class. One of our goals is therefore to identify as many PL classes as we possibly can. When identifying PLs incorporated in the current pipeline we essentially perform a targeted analysis. Yet, we also spot unknowns which may represent relevant differences between the investigated groups which can be further investigated. In the current method, it is fairly easy to add new (phospho)lipid classes based on their chemical composition and physico-chemical properties.

The method works well for low-resolution data as it addresses and aids to resolve ambiguous phospholipid assignments. PG and BMP, for example, are two classes of PLs that have the exact same chemical formula and therefore the same m/z. Using our algorithm in combination with appropriate chromatographic separation and internal standards achieves the correct identification of compounds from each class. Another example is PE and pPE, which differ in chemical structure but at low resolution cannot be distinguished based on m/z alone. In this case, the difference in polarity causes a distinct shift in the pattern of the peaks observed for each of these clusters, so that the ambiguity can be resolved and PE and pPE can be distinguished. A third example is PI and PS, which often co-elute. Since PSs have even masses and PI have odd masses in ionized state, the first isotope of one species often overlaps with the monoisotopic peak of the other species. Identification followed by isotope correction can then solve this problem and annotate species of both PL classes.

For high-resolution data, the accuracy of the observed m/z values is higher and the peaks are much narrower on the m/z-axis, so that there is much less overlap between different
peaks. This results in a much greater number of peak groups, but the percentages of identified metabolites are still comparable to what we find for low-resolution data (data not shown). At high resolution, the benefit of this method is in the larger number of peaks that can be identified in an automated fashion, without (or with minimal) human intervention.

**Material and Methods**

**Chemicals**
Betulinic acid (BioSolution Halle, Germany, >99% purity) was dissolved in DMSO at 4 mg/ml and stored at -80°C.

**Cell culture**
HeLa cells were obtained from the ATCC. Cells were cultured in IMDM supplemented with 8% FCS, 2 mM L-glutamine, 40 U/ml penicillin and 40 µg/ml streptomycin.

**Mass spectrometry**
Cell pellets were resuspended in water and sonicated for 30 s (8 W) using a tip sonicator. Protein concentration of the homogenates was determined according to the BCA protocol(14). Phospholipids were extracted using a single-phase extraction. A defined amount of internal standards (0.1 nmol of CL(14:0)4, 0.2 nmol of BMP(14:0)2, 2.0 nmol of PC(14:0)2, 0.1 nmol of PG(14:0)2, 5.0 nmol of PS(14:0)2, 0.5 nmol of PE(14:0)2, 1.0 nmol of PA(14:0)2, 2.0 nmol of SM(14:0)2, 0.02 nmol of LPG(14:0), 0.1 nmol of LPE(14:0), 0.5 nmol of LPC(14:0), 0.1 nmol of LPA(14:0) (purchased from Avanti Polar Lipids, Alabaster, AL, USA), dissolved in 120 µL of chloroform/methanol (1:1, v/v) and 1.5 mL of chloroform/methanol (1:1, v/v) were added to cell homogenates that contained 1 mg of protein. Subsequently, the mixture was sonicated in a water bath for 5 min, followed by centrifugation at 15,000 x g for 5 min. The organic layer was transferred to a glass vial and evaporated to dryness under a nitrogen steam at 60°C. Subsequently, the residue was dissolved in 200 µL of chloroform/methanol/water (50:45:5, v/v/v) containing 0.01% of NH4OH, and 20 µL of the solution was injected into the liquid chromatography-mass spectrometry (LC/MS) system. LC/MS analysis was performed as described in (15). In negative mode, mass spectra of phospholipid molecular species were obtained by continuous scanning from m/z 380 to m/z 1100 with a scan time of 1 s. In positive mode, mass spectra of PC, LPC and SM were obtained by continuous scanning for product ions with m/z 184 from m/z 400 to 1000. Mass spectra for PE, LPE and PE plasmalogens were obtained by scanning on neutral losses of 141 from m/z 400 to 1000. The raw LC/MS data were converted to mzXML format using msConvert (16) for the negative scan data and ReAdW (17) for the positive specific scan data.
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Data pre-processing

All LC-MS spectra of the samples were pre-processed with XCMS (11). In brief, in the first step all peaks in the individual LC-MS spectra were found using the matched filtration method. The XCMS function ‘matchedFilter’ was used with parameters fwhm=11, max=5, snthresh=7, steps=2, profMethod=Intlin, step=0.2, mzdiff=0.2. Some spectra from the negative scan mode contain neighboring or partly overlapping peaks (e.g., for PG and BMP) that have identical m/z values and similar retention times. To detect such peaks we modified the matched filtration method by recalculating the second-order derivative Gaussian filter after the integration of each peak within the same m/z slice. For the precursor ion scans, we minimized the number of false positive peaks by retaining only peaks with at least three consecutive points above the signal-to-noise threshold in the peak list.

After peak finding, peaks from different samples were matched and grouped together into a peak group, while accounting for small variations in m/z and retention time. The XCMS ‘group’ function was used with parameters family=symmetric and smooth=linear. Subsequently, chromatograms from the samples were aligned with the function ‘retcor’ (minfrac=0.80, bw=2). In the last step of XCMS, a table was generated comprising the peak groups, with median mass and retention time calculated from all samples.

Assignment of compound names

For the identification of metabolites, the assignment of compound labels from a certain class of PL was limited to a subset of peak groups, contained within a box spanning a specific m/z and retention time range, covering the whole PL cluster. Subsequently, a line was defined (as shown in Figure 3a) that runs through the cluster of peak groups. Compound names were then assigned to peak groups that are in close proximity to the line. Duplicate assignments were resolved based on the likelihood of each separate assignment to occur, taking into account the patterns of peaks. The steps in the assignment are explained in more detail below:

   Step 1. Generation of an m/z look-up table for lipid compounds;
   Step 2. Assignment of labels within a PL cluster;
   Step 3. Collecting all peak groups;

Step 1. Generation of an m/z look-up table for PL compounds. A look-up table, containing the chemical composition and corresponding masses (m/z) in positive and negative scan modes for all theoretically possible combinations of chain length and number of double bonds for every class of PL, was generated. For a single fatty acid side chain, the chain lengths ranged from 14 to 28, and the number of double bonds ranged from 0 to 6; multiples of these were used for PLs with more than one variable side chain. For each chemical
structure, the expected isotope percentages were calculated using the R package Rdisop (18) and added to the table.

**Step 2.** Assignment of labels within a PL cluster. A plot of median m/z versus median RT for all peak groups (Figure 2) was used to determine the location of the clusters of peak groups that originated from the same class of PL. For each PL class, an m/z and RT box was defined based on visual inspection of the plot and a line through two peak groups in the cluster was used to define the location and direction (slope) of the peak groups in that particular cluster (Figure 3). Assignments of PLs within a cluster were done by matching the m/z of each peak group to the masses in the compound look-up table, allowing for small deviations (±0.2 atomic units for the data set described in this paper, or ±3 ppm for high-resolution data). The context of the peaks was also incorporated in the assignment step: checking for systematic patterns in lines of compounds with the same chain length or degree of saturation (Figure 3B). Whenever more than one assignment within the same class was possible, (e.g., PC(37:0) or PC(38:7), both with m/z 804.6), the most likely assignment was chosen. For the assignment of peaks from doubly-charged molecular species (e.g. CL and mICL), only peaks with isotope peaks at m/z ± 0.5 were considered.

**Step 3.** Collecting all peak groups. The lists of peak groups for each PL class, assigned in Step 2, were collated. Multiple assignments for one peak (e.g., PC(38:7) or pPC(39:6), m/z 804.6), were resolved based on the patterns of the peak groups in the different clusters. All assigned peak groups were then mapped back onto the original peak group list, so that the full list contains a combination of assigned and non-assigned peaks.

**Step 4.** Manual correction of incorrect assignments. By investigating the assignments in the close-up plots that were generated for every PL cluster (Figure 3B), erroneous or suspicious assignments were edited and corrected. Generally, about 95% of the assigned peak groups were identified correctly by the pipeline.

*Data processing*

After the identification of the monoisotopic peaks for different PLs, their isotope peaks were marked in the peak group list. Intensities for observed isotope peaks were corrected using the method described by (19) in order to obtain deconvoluted intensities for peak groups which overlap with isotope peaks from other compounds. For normalization purposes, intensities of peaks belonging to a certain PL class were scaled to the response of the IS of that class. Thus, the intensity of the IS peak was set to the corresponding concentration of that IS that was added to each sample, and the intensities of the other peaks of the same PL class could be estimated semi-quantitatively, relative to that of the IS peak. Lastly, the intensities of all peaks were normalized to the protein concentration of the homogenate.
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The result of the pre-processing pipeline as described, is a table in Excel format that contains all measured peak groups, characterized by their median m/z and RT values and, where possible, their identified compound name (see supplemental files 1-4). Isotope-corrected, normalized intensities and per-feature statistics based on the normalized and corrected values are included in the table, as well as EIC plots for the peak areas and boxplots, to allow for quick visual assessment of the various peaks.

Acknowledgement/grant support

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Improved identification of lipids using physico-chemical properties

References


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Supplementary figures/tables

**Supplementary figure 1: Heatmap of differences in BMP and PG composition.** (A) The BMPs have been sorted by total number of double bonds in the combined side chains. (B) The PGs have been sorted by total number of double bonds in the combined side chains. In both heatmaps no clear distinction between DMSO and BetA treated samples are observed.

**Supplemental table I: Number of PLs identified per major class in Negative scan mode**

<table>
<thead>
<tr>
<th></th>
<th>Monoisotopic peaks</th>
<th>Monoisotopic + isotope peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td>CL2</td>
<td>69</td>
<td>147</td>
</tr>
<tr>
<td>mlCL2</td>
<td>32</td>
<td>62</td>
</tr>
<tr>
<td>PA</td>
<td>12</td>
<td>33</td>
</tr>
<tr>
<td>PE</td>
<td>29</td>
<td>53</td>
</tr>
<tr>
<td>PG</td>
<td>23</td>
<td>43</td>
</tr>
<tr>
<td>PI</td>
<td>51</td>
<td>108</td>
</tr>
<tr>
<td>pPE</td>
<td>16</td>
<td>27</td>
</tr>
<tr>
<td>PS</td>
<td>23</td>
<td>48</td>
</tr>
</tbody>
</table>

*In full negative mode, 285 out of 2163 (13%) peak groups can be identified as monoisotopic and 571 out of 2163 (26.4%) can be identified as monoisotopic plus isotope peaks. Numbers in the Results section refer to a truncated list, where the weakest peak groups have been omitted to improve the clarity of Figure 2.*
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**Supplemental table II**: Number of PLs per major class identified in Positive scan mode (PR184)

<table>
<thead>
<tr>
<th></th>
<th>Monoisotopic peaks</th>
<th>Monoisotopic + isotope peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>66</td>
<td>151</td>
</tr>
<tr>
<td>pPC</td>
<td>23</td>
<td>45</td>
</tr>
<tr>
<td>aPC</td>
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<td>11</td>
</tr>
<tr>
<td>LPC</td>
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<td>58</td>
</tr>
<tr>
<td>LpPC</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>SM</td>
<td>22</td>
<td>42</td>
</tr>
</tbody>
</table>

**Supplemental table III**: Number of PLs identified per major class in Positive scan mode (NL141)

<table>
<thead>
<tr>
<th></th>
<th>Monoisotopic peaks</th>
<th>Monoisotopic + isotope peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>39</td>
<td>89</td>
</tr>
<tr>
<td>pPE</td>
<td>13</td>
<td>24</td>
</tr>
<tr>
<td>aPE</td>
<td>17</td>
<td>27</td>
</tr>
<tr>
<td>LPE</td>
<td>10</td>
<td>14</td>
</tr>
</tbody>
</table>

*a* In full PR184 scan mode, 144 out of 785 (18.3%) peak groups can be identified as monoisotopic and 316 out of 785 (40.2%) can be identified as monoisotopic plus isotope peaks. In full NL141 scan mode, 74 out of 225 (32.9%) and 154 out of 225 (68.4%) can be identified as monoisotopic plus isotope peaks. Numbers in the Results section refer to a truncated list, where the weakest peak groups have been omitted.

**Supplemental table IV**: Clusters of PLs that can be identified using the algorithm

<table>
<thead>
<tr>
<th>One variable side chain</th>
<th>Two variable side chains</th>
<th>Three variable side chains</th>
<th>Four variable side chains</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPC, LPG, LPS, LPE, LPI, LPA, SM, ST, STOH, LpPE, LpPC, LaPE, LlyPG, Cer, FA, FAOH</td>
<td>PC, diLPC, diLpPC, diSM, diSMCl, diLPE, diLpPE, PG, PS, PE, PI, PA, BMP, pPE, aPE, pPC, aPC, pPA, aPA, diCL1, diCL2, mINAPS1, mINAPS2, lyPG, PIP, diFA, DAG</td>
<td>mICL1, mICL2, NAPS2, NAPS1, SLBPA, TAG, triLPE</td>
<td>CL1, CL2, CL2OH, diPG, diPE, diPA, dipPE, diPCCl, diPS, diPC, dipPC</td>
</tr>
</tbody>
</table>

*a* LPL; lyso-PL, pPL; plasmalogen PL, aPL; O-alkyl PL, diPL; dimeric PL, mIPL; monolyso-PL, FA; fatty acid, NAPS; N-acetyl PS, suffix 1 (as in mICL1); singly charged, suffix 2; doubly charged, suffix OH; hydroxy, suffix Cl; chloride adduct. SLBPA; semilysobisphosphatidic acid (a.k.a. hemi-BMP), DAG; diacylglycerol, TAG; triacylglycerol.
Chapter 6

General Discussion

“Curiouser and curiouser!”

Lewis Carroll, *Alice in Wonderland*
The role of autophagy in BetA-induced cell death

Since the discovery of BetA as an anti-cancer compound, research has focused on finding the mechanism of action of this natural compound. Research quickly showed that the mitochondria were involved and apoptosis was induced, although independent of BAK/BAX.\(^1\) When a caspase inhibitor was used, these compounds did not prevent cell death, indicating that alternative, caspase-independent cell death pathways must be activated. In chapter 2, we describe that necroptosis is not induced, however a massive and rapid form of autophagy is induced. This induction of autophagy could be blocked by cyclosporine A, an inhibitor of the permeability transition pore. Blocking the opening of this pore by cyclosporine A resulted also in a block in apoptosis as previously described.\(^2\) This observation suggests that autophagy is a consequence of mitochondrial damage triggered by BetA and can be prevented by inhibition of PT-pore opening. We also observed that in autophagy knock out cells, enhanced cell death was observed even when apoptosis was blocked by caspase inhibitors. These data suggest that autophagy acts as a survival mechanism rather than the cell death execution machinery and that there must be a yet undefined other cell death mechanism involved that is induced by BetA. Alternative pathways to death have been reported and could for instance involve lysosomal permeabilization. Interestingly one of the compounds that is reported to induce lysosomal destabilization, siramesine, shares some properties with BetA, as it also induces cell death independent of caspases, BCL-2 and P53\(^{3-5}\) pointing to a potential mechanistic overlap.\(^4,5\)

Lysosomal membrane permeabilization can be measured using fluorescent labeled galectin-1 or galectin-3 cells\(^6\) and experiments to test the role of the lysosome in BetA-induced cell death should be conducted. It is important to note though that we have shown that inhibition of cathepsin B, which is reported to be crucial for the lysosomal destabilization \(^7\), did not influence BetA-induced cell death, arguing against lysosomal membrane destabilization as the causal pathway to cell death. Therefore more work needs to be performed and we decided to focus more in detail on how the mitochondrial BetA-induced cell death occurred.

Differential effects of BetA on cancer cells and normal cells

One characteristic of BetA is that its cytotoxic effects are observed on cancer cells and not on healthy cells.\(^6,9\) For a long time it has remained enigmatic as to why this differential effect is observed. In chapter 3, we described that interfering with the fatty acid metabolism of a cancer cell, by inhibiting stearoyl CoA desaturase enzyme, the side chains of the phospholipid cardiolipin become more saturated, which results in morphological changes in the inner membrane of the mitochondria followed by cell death. We also confirmed that the cytotoxic effect of BetA is only on cancer cells and not on healthy cells. We believe these observations could explain the selectivity for cancer cells as a hallmark of cancer is altered metabolism. That is, cancer cells have a different lipid metabolism as compared to healthy
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cells. Cancer cells increase their \textit{de novo} fatty acid synthesis, to fulfill the need for new lipids in order to create new biomass which is needed even under scarce conditions. It is energetic very inefficient to generate \textit{de novo} fatty acids while enough fatty acids can be taken up by fatty acids transporters from the surrounding suggesting that cancer cells benefit from \textit{de novo} fatty acids for yet undefined reasons. The end product of \textit{de novo} fatty acid synthesis is palmitic acid. This saturated fatty acid is converted to unsaturated fatty acids by SCD-1 and further elongated by several elongation enzymes to create longer fatty acids. In several types of cancer, SCD-1 is overexpressed.\textsuperscript{[10]} Cancer cells have become addicted to the constant rate of conversion of saturated fatty acids into unsaturated fatty acids and thereby the levels of SCD-1 they express. BetA and SCD-1 inhibitors affect this balance and thereby create a toxic pool of saturated fatty acids. Importantly, in healthy cells basal levels of saturated fatty acids are lower as compared to tumor cells\textsuperscript{[11]} as normal cells do not utilize the \textit{de novo} synthesis pathway. Instead, healthy cells take up unsaturated fatty acids from the surrounding mainly provided by food intake. Thereby inhibiting the activity of SCD-1 shows less impact and is therefore less harmful to healthy cells as they are not dependent on this pathway (described in chapter 3).\textsuperscript{[12]} Unsaturated fatty acids are important precursors for various products in the cell, like phospholipids (cell membrane), diacylglycerols (signaling) and triglycerides (energy storage).\textsuperscript{[13]} Overexpression of SCD-1 is a result of the overall increase of \textit{de novo} fatty acid synthesis where besides SCD-1 also sterol regulatory element binding proteins (SREBPs) are overexpressed.\textsuperscript{[14, 15]} SREBPs control the expression of several enzymes (ATP-citrate lyase (ACL), ACC and FASN) required for endogenous cholesterol, fatty acid (FA), triacylglycerol and phospholipid synthesis. Upregulation of these enzymes demands the cell also to increase their SCD-1 activity to fulfill the need of an unsaturated fatty acid pool.

The \textit{de novo} fatty acid synthesis pathway is a potential drug target due to the differential usage of this pathway between normal cells and cancer cells. Several studies have shown that inhibition of fatty acid synthesis enzymes by inhibiting FASN, ACC or ACL results in cancer cell death \textit{in vitro} and retards the growth of tumors \textit{in vivo}.\textsuperscript{[16-20]}

Also shown in chapter 3 is that in cancer cells the saturation levels of the side chains of cardiolipin are already more saturated as compared to healthy fibroblasts. BetA and SCD-1 inhibitors treatment result in an even higher saturation grade of the side chains of cardiolipin. Saturated fatty acid makes membranes more rigid and loss of membrane fluidity results in loss of function and structure and we hypothesize that this results in the inner cristae morphology change, which results in permeability pore opening and leakage of cytochrome c into the cytosol. This initial higher saturation grade of membranes makes cancer cells more vulnerable for inhibition of SCD-1 activity as compared to normal cells. Barth syndrome patients also have highly saturated fatty acid side chains of cardiolipin and similar morphological changes in the mitochondria are observed.\textsuperscript{[21, 22]} However these modifications by itself are not sufficient for cytochrome c leakage to occur as most cells in
Barth syndrome patients show no increase in cell death. This suggests that besides saturation of cardiolipin side chains, additional changes are needed to induce cell death. This could be reactive oxygen species (ROS), as inhibiting mitochondrial complex 1 results in OPA1 (a GTPase in the inner mitochondrial membrane which plays a role in regulating mitochondrial fusion and pro-apoptotic remodeling of the mitochondria) desoligomerization leading to ROS production. This loss of oligomerization of OPA1 results in cytochrome c release and mitochondrial cristae remodeling. It is known that cancer cells have higher levels of ROS and therefore it could be that the combination of higher saturation of cardiolipin and the high amounts of ROS induced in cancer cells results in cell death.

It is likely that due to the increased need of lipids, cancer cells also has more lipid droplets which they can use for their supply of fatty acids. During nutrient deprivation, triglycerides in lipid droplets are hydrolyzed into fatty acids. The breakdown of these stored lipid droplet products has been shown to occur, besides cytosolic hydrolytic enzymes or lipases, via a specialized form of autophagy, named lipophagy, which link autophagy to lipid metabolism. Lipophagy may protect cells for apoptosis by breaking down lipids to supply for energy and prevention of ATP depletion. Another protective role for lipophagy is by preventing liver injury induced by increased hepatic lipid accumulation or steatosis. Lipids may also play a role in the regulation of levels of autophagy. It was shown that unsaturated fatty acid, oleic acid, promoted the formation of triglyceride-enriched lipid droplets and induced autophagy, while saturated fatty acid, palmitic acid, was poorly incorporated into lipid droplets and suppressed autophagy and rather increased apoptosis. This suggest that formation of lipid droplets and induction of lipophagy is protective for cells.

The differential effect of BetA on cancer cells and normal cells could also be due to massive induction of autophagy as a response to the induced mitochondrial stress. The autophagy induced by BetA could also be partially lipophagy, to increase the amount of unsaturated fatty acids to prevent the toxic effect of accumulating saturated fatty acids by the inhibition of SCD-1. Due to the increase of saturated fatty acids, the protective role of lipid droplets and autophagy are blocked resulting in increased cell death. As normal cells have higher amounts of unsaturated fatty acids as compared to cancer cells it could be that BetA induces more autophagy and lipophagy in cancer cells to overcome the toxic effects of saturated fatty acids. Investigating the role of lipophagy and the blocking of lipophagy, more in detail could result in potential new targets. Combination of blocking lipophagy and SCD-1 inhibition might result in a faster more efficient cell death.

The role of cardiolipin in cell death

The toxic saturated fatty acids are incorporated in several phospholipids and one phospholipid that shows dramatic changes in saturation levels is cardiolipin. Cardiolipin is a phospholipid that is important for the structure of the inner membrane of the mitochondria.
and it is involved in apoptosis. Two main levels of apoptosis regulation by cardiolipin have been described. The first involves the release of cytochrome c in a BAK/BAX dependent fashion. Cytochrome c interacts with cardiolipin in the outer leaflet of the IMM through two independent binding sites, this is needed for the function of cytochrome c as an electron carrier in the mitochondrial respiratory chain.\textsuperscript{(29-32)} Cardiolipin-bound cytochrome c acts as a peroxidase capable of catalyzing H2O2-dependent peroxidation of cardiolipin. This cardiolipin oxidation is an essential step in the release of cytochrome c during apoptosis.\textsuperscript{(33)}

The second level of cardiolipin-dependent apoptosis regulation that is suggested to occur, involves Bid-induced cytochrome c release. During apoptosis, Bid is cleaved by caspase 8 to produce tBid which translocates to the mitochondria. tBid has been suggested to bind to CL-enriched contact sites and induces the translocation of BAK and BAX to the mitochondrial outer membrane.\textsuperscript{(34-36)}

Cardiolipin is therefore suggested to serves as a platform to allow apoptosis via the BCL2 family to occur. Whether cardiolipin saturation levels change this platform function is not clarified, but is a possibility. The level of saturation is however clearly of importance to the physiological function of cardiolipin and lipids in general. In biological membranes one finds unsaturated fatty acids of which the structural properties allow for membrane fluidity. Cardiolipin has four acyl side chains that are highly unsaturated to maintain normal cellular function.\textsuperscript{(34)} These side chains can be remodeled via tafazzin. We hypothesized that BetA besides SCD-1 inhibition also increases the turnover of lipids and thereby the incorporation of saturated fatty acids into cardiolipin. As cardiolipin regulates and is involved in permeability pore opening \textsuperscript{(34, 37)} we hypothesized that the saturation of cardiolipin results in morphological and functional changes in the mitochondrial cristae and leads to permeability pore opening and cytochrome c release.

The mitochondrial network is maintained by a continuous process of fusion (elongation of mitochondria) and fission (fragmentation of mitochondria). Whether fusion/fission is important for cytochrome c release is still debated, however during apoptosis mitochondria are remodeled via activation of the fission machinery and synchronal neutralization of the fusion machinery.\textsuperscript{(38, 39)}

Mitochondrial fission occurs at the same time as the activation of BAX, mitochondrial outer membrane permeabilization and the resulting cytochrome c release.\textsuperscript{(40)} On top of that, it was shown that disruption of OPA1 complexes results in cytochrome c release and mitochondrial cristae remodeling.\textsuperscript{(23)} These data indicate that mitochondrial fission/fusion and cytochrome c release are linked to each other. Cardiolipin also plays a role in mitochondrial fission/fusion\textsuperscript{(34)} and therefore an increase in saturation grade of the cardiolipin side chains could result in the observed mitochondrial fragmentation. How this fragmentation is induced by cardiolipin needs further investigation. Cardiolipin side chain
remodeling into more saturated is therefore a very interesting mechanism for further investigation and possible therapeutic targets.

The role of SCD-1 in cancer therapy

The differential effects of both BetA and SCD-1 inhibition on healthy cells and tumor cells makes both compounds very interesting to investigate further for therapeutic applications. In the past pharmaceutical companies started to look for small molecule SCD-1 inhibitors to treat metabolic diseases like obesity, diabetes and fatty liver as these diseases are associated with up-regulation of SCD-1. Since 2005 several patents have been granted and many of these compounds have good in vitro activity and pharmacodynamics in rodent models, unfortunately, only a few of these inhibitors have progressed to clinical trials and no reports can be found of compounds beyond phase Ila studies for obesity, diabetes of fatty liver disease treatment. The differences of lipid metabolism in rodents to humans is given as the main explanation.[41] Research in the last years have shown that SCD-1 plays a role in the regulation of metabolism and growth signaling pathways in cancer. SCD-1 contributes to maintain a shift in lipid metabolism and intracellular signaling (i.e. activation of Akt signal and deactivation of AMPK pathway). This results in an accelerated rate of cell proliferation, increased invasiveness and enhanced survival.[10] Several studies have shown that inhibition of SCD-1 (either genetically or pharmaceutical) leads to reduced cancer formation in vivo and transformation in vitro.[10,42] The mechanism by which SCD-1 inhibition leads to tumor reduction has been unclear. A hypothesis mentioned in literature, is that changes in lipid composition result in ER stress and trigger unfolded protein response (UPR).[43]

We show that inhibition of SCD-1 by chemical inhibition (BetA and SCD-1 inhibitor) leads to increased saturation of CL. This saturation leads to morphological changes in the inner membrane of the mitochondria and results in cell death. How exactly the changes in saturation and morphology results in cell death remains unclear, however we hypothesize that the change in the inner membrane results in opening of the PT-pore and thereby release of cytochrome c into the cytosol. (chapter 3) In our research we have not tested ER stress and UPR. It could be that the ER stress inflicted by shifting the balance between saturated fatty acids and unsaturated fatty acids precedes the mitochondrial stress we observe.

Currently no clinical trials are ongoing for SCD-1 inhibition (clinical trial.gov) in the treatment of cancer. Inhibition of SCD-1 as monotherapy could not be optimal as we observed that inhibition of SCD-1 results in a slower cell death as compared to BetA. Therefore the focus should be more on combination therapy, where besides SCD-1 inhibition another stressor is applied, resulting in synergy of both compounds. We have tried to find this combination of SCD-1 inhibitor and a compound that results in the same efficacy and efficiency as BetA-induced cell death. However several compounds that
stressed the mitochondria did not result in cell death synergy when combined with SCD-1 inhibition. Further research into understanding BetA mechanism and synthetic lethality using SCD-1 inhibitor could lead to a beneficial non-toxic combination therapy.

**Betulinic acid as cancer therapy**

Besides looking into SCD-1 inhibition for therapeutic approaches, BetA also has some potential. Research by others and previously by our group has shown BetA has no toxic effects *in vivo*.\(^8,^9\) The development for BetA as a potential drug is not as advanced as SCD-1 inhibitors, however the higher efficacy of BetA makes it a better compound as compared to SCD-1 inhibitors. Unfortunately, BetA is a very lipophilic compound and therefore harder to administer to patients. It is known that lipophilic drugs can be more toxic because they bind (i.e to receptors, plasma, proteins or tissue) while unbound drugs most of the time are responsible for the efficacy of the drug.\(^44,^45\) For lipophilic compounds, there are usually much higher concentrations of bound drug than unbound drug, therefore toxicity will be more likely if it is determined by total drug concentrations as higher doses are needed to get enough unbound drug in the blood system. However up to now, even when high doses of BetA were used *in vivo* no toxic effects were observed in normal tissue although the formulations used are not suited for the clinic.\(^8,^9\) Accordingly there is a need for a non-toxic formulation which can deliver BetA, preferably specific, to the tumor.

**Cancer stem cells targeted by BetA**

The cancer stem cell model hypothesizes that cancers are organized into a hierarchy of subpopulations of tumorigenic cancer stem cells and their non-tumorigenic progeny.\(^46-48\) Cancer stem cells are defined as a subset of tumor cells which possess self-renewal and multi-lineage differentiation potential.\(^49,^50\) Cancer stem cells are known to be more resistant to conventional treatment due to their (acquired) resistance mechanisms.\(^51,^52\) In chapter 4 we show that BetA can induce a rapid cell death in colon cancer stem cells. We hypothesize that this induction of cell death is SCD-1 dependent as SCD-1 inhibition also kills colon cancer stem cells, although the induction takes longer than BetA. The precise mechanism by which BetA induces cell death in colon cancer stem cells is still unclear and further research should reveal if indeed BetA results in SCD-1 inhibition and a saturation of CL in these cells leading to apoptosis. Due to the rapid induction of cell death in colon cancer stem cells as compared to cancer cells a different mechanism might be involved. It could be that colon cancer stem cells rely more on their fatty acids as they possess more lipid droplets than regular cancer cells.\(^53\) Interference in the unsaturation grade of these fatty acids could be a weak spot for these stem cells. Cancer stem cells protect themselves from classical chemotherapy by using BCL-XL-dependent mechanisms \(^54\), as BetA-induced killing is independent of the BCL-2 family this could be a reason why BetA targets the cancer stem cells. How CSCs die exactly remains to be elucidated. However these data suggest that CSCs
could be targeted very efficiently if the precise mechanism of action of BetA in these cells is known.

Lipidomics as a tool for diagnostics and monitoring of patient.

Studying lipids is complex due to the diversity of many lipids. Analysis of lipids was traditionally done by thin-layer chromatography, gas chromatography and mass spectrometry. Recent years more and more technical advances in mass spectrometry have created the ability to study lipids in a metabolomics way the so-called lipidomics. Lipidomics aims to study the pathways and networks of lipids by the characterization and quantification of all lipids in a sample.\(^{[55]}\) In chapter 5 we described a new lipidomics pipeline which we have tested for BetA treated samples. We show that using the lipidomics pipeline we can identify and separate a lot of lipids based on their features i.e. their saturation grades. We show that we can distinguish samples that are DMSO or BetA treated based on their CL saturation grade. This could be used in the clinic to discriminate healthy individuals from Barth syndrome patients, but also if SCD-1 inhibitor of BetA makes it into the clinic this pipeline can be used to monitor treatment response. This method could be used for monitoring treatment response of compounds that have BetA and/or SCD-1 inhibitor mode of action or monitor the effect of SCD-1 inhibitors and/or BetA when it makes it into the clinic. With further optimization of the lipidomics (i.e. improved high resolution mass spectrometry which result in better detection of masses with a higher accuracy and thus leading to more reliable identification of lipids) this pipeline could also be used for validation of new compounds to see if they exert the mechanism to inhibit SCD-1 and thereby increasing the saturation of CL.

Besides playing a role in cancer, lipids and their metabolism are also involved in several other diseases.\(^{[55, 56]}\) This makes lipidomics a great new tool for diagnostics. The ultimate goal of lipidomics would be to have one patient sample and measure the complete lipidome in a fast and accurate fashion so the diagnosis of patient disease could be made. Besides diagnosis, monitoring of disease progression, treatment response and biomarkers research are possible.

Concluding remarks

There are still a lot of things we do not understand about BetA and how it induces cell death. The observations that when apoptosis is blocked by caspase inhibitors, BetA still induces cell death indicates that a different form of cell death or even a new pathway remains to be elucidated. And up to now due to its lipophilic character no clinical trials are attempted. In this thesis we provide data that contribute to the understanding of BetA-induced apoptotic cell death. The finding that inhibition of SCD-1 by BetA results in saturation of cardiolipin and cytochrome c release opens a new field for cancer research and potential new use for SCD-1 inhibitors in the clinic. Targeting cancer stem cells is a kind of Holy Grail in cancer
research, as eradicating all tumor cells is wanted. Interestingly, applying BetA on colon cancer stem cells resulted in a massive and fast cell death in these otherwise therapy resistant cells. Discovering the precise mechanism of action of BetA in these cells will provide the cancer field with potential new drug targets a possible therapy for hard to target cancers. Monitoring drug response can be done by using the lipidomics pipeline described in this thesis and this could result in faster diagnosis and specialized patient care. Taken together, the new findings in this thesis can provide a basis for future studies for cancer therapy based on cancer metabolism and especially SCD-1 inhibition and cardiolipin saturation and on the quest of the mechanism of action of BetA.
References


Chapter 6


40. Martinou JC, Youle RJ. Which came first, the cytochrome c release or the mitochondrial fission? Cell DeathDiffer. 2006;13(8):1291-5.


Discussion
Chapter 6


Annexes
“Well that was the silliest tea party I ever went to! I am never going back there again!”

*Lewis Carroll, Alice in Wonderland*
Mechanistic insight into BetA-induced cell death

This thesis focuses on the mechanisms of BetA induced cancer cell death. In chapter 1, we start with an introduction on cancer, cancer therapeutics, cell death mechanisms and BetA. The mechanism by which BetA induces cell death has been investigated by many people over the past years, however the exact mechanism remained unclear. In chapter 2 we explore the possibility of other cell death mechanisms besides apoptosis. We observe that BetA induces cell death in cells which are treated with a caspase inhibitor indicating that another cell death mechanism must be causing the observed cell death. We therefore investigated the role of necroptosis in BetA-induced cell death. Cells treated with caspase inhibitor and necrostatin-1 (a necroptosis inhibitor) also showed induction of cell death. So we proceeded to another possible cell death mechanism, autophagy. Upon BetA treatment massive induction of autophagy, as shown by lipidated LC3II, was observed both by confocal microscopy as well as western blot. Autophagosomes needs to be degraded by the lysosomes in order to get full breakdown of the cargo. Autophagic flux was measured using long lived proteins, a tandem RFP/eGFP-tagged LC3 protein and a tandem mCherry/GFP-tagged p62 protein. Previously, we observed that cyclosporin A, which targets cyclophilin D and effectively serves as an inhibitor of the mitochondrial permeability pore, prevents BetA-induced apoptotic features. We therefore also investigated the role of this compound in the induction of autophagy and showed that autophagosome formation was significantly reduced when cells were pre-treated with cyclosporin A. These data suggest that BetA-induced autophagy is a consequence of mitochondrial damage triggered by BetA and can be prevented by inhibition of PT-pore opening. Autophagy, even though initially regarded as a cell survival pathway, can play a role in dying cells as well and has been suggested to serve as a balancing mechanism between cell survival and cell death. To test the role of autophagy in BetA-induced cell death we used autophagy-deficient cells. Surprisingly, we observed an increase in BetA-induced cell death in these cells, which could not be prevented by caspase inhibition. Both autophagy-proficient and -deficient cells displayed a similar increase in mitochondrial depolarization, suggesting that the initial mitochondrial insults were independent of autophagy induction. Taken together these data indicate that autophagy serves primarily as a survival mechanism in BetA treated cells and that BetA induced cell-death is independent of autophagy, necroptosis and caspasates but requires the mitochondria.

How the induction of apoptosis was realized was therefore further investigated in chapter 3. When analyzing the mitochondria via confocal or electron microscopy we observed mitochondrial morphology changes upon BetA treatment. The mitochondrial tubular network became fragmented and the mitochondria displayed a circular morphology by electron microscopy. These circular cristae are also detected in patients with a X-linked syndrome called Barth syndrome, which suffer from a mutation in Taffazin gene. Taffazin plays a role in cardiolipin remodeling and side chain saturation and cells of these patients
display enhanced cardiolipin saturation that causes mitochondrial ultrastructural as well as functional abnormalities. We therefore investigated the saturation grade of cardiolipin in cells treated with BetA and show that upon BetA treatment cardiolipin side chains get more saturated as compared to vehicle treated cells. To investigate how this saturation occurs we looked into the function of stearoyl CoA desaturase 1 (SCD-1) a key enzyme that converts saturated fatty acids into unsaturated fatty acids. We observed in several cancer cell lines that the activity of SCD-1 was blocked by BetA, resulting in an increase in saturated fatty acids. The role of SCD-1 inhibition in cardiolipin saturation was further corroborated by the use of specific SCD-1 inhibitors. In addition, exogenously added fatty acids (unsaturated or saturated) strongly affected the saturation grade of cardiolipin. When we treated cells with BetA or SCD-1 inhibitor in combination with the saturated fatty acid, palmitate, we observed an increase in saturation grade of cardiolipin. In contrast, when using the unsaturated fatty acid, palmitoleic acid, cardiolipin saturation decreased. Importantly, the BetA or SCD-1 inhibitor-induced mitochondrial morphology, cytochrome c release, cell death and clonogenic growth were all strongly affected by exogenously added fatty acids, confirming that the inhibition of SCD-1 activity is instrumental in inducing cell death.

Previously we showed that normal cells are less affected by BetA and this is consistent with our current findings that revealed no morphology changes in mitochondria in normal fibroblasts upon BetA treatment. As these cells have less SCD-1 activity, but more importantly do not appear to depend on this activity to acquire unsaturated fatty acids, the inhibition of SCD-1 by BetA or SCD-1 inhibitor is not as detrimental as compared to tumor cells. Taken together these data indicate that cancer cells carry an intrinsic mitochondrial vulnerability that can be effectively targeted by inhibition of SCD-1, using either specific inhibitors or BetA, and is not observed in normal fibroblasts. This vulnerability depends on enhancing the saturation of cardiolipin leading to altered mitochondrial structure and as a consequence mitochondrial leakage and cell death.

Cancer stem cell sensitivity

Cancer stem cells are known to be very resistant to therapy. As BetA is acting in a broad fashion to kill cells we investigated the effect of BetA on colon cancer stem cells. In chapter 4 we show that BetA induces a rapid cell death in three different colon cancer stem cell lines in a time and concentration dependent fashion. Strikingly already after 2 hours an induction of cell death is observed. As the spheroid cultures contain both cancer stem cells and more differentiated cells, we set out to determine if BetA induces cell death in the cancer stem cell population using FACS-based sorting. Using this approach we revealed that BetA is capable of inducing cell death in both differentiated tumor cells and cancer stem cells. This was mirrored in an almost complete loss of clonogenic capacity of these cancer stem cell cultures treated with different concentrations of BetA. Already after 2 hours of treatment with a low concentration of BetA, clonogenic capacity is reduced in GTG7 cells, while the
other lines require slightly higher BetA concentrations. In the previous chapter we showed that BetA induces cells death via inhibition of SCD-1. We therefore also investigated the role of SCD-1 in inducing cell death in colon cancer stem cells. Although it takes more time to induce cell death with SCD-1 inhibitors in these cells as compared to BetA, the induction of cell death and loss of clonogenic capacity is very efficient. We therefore conclude that SCD-1 is a crucial player in the maintenance of stemness of cancer cells and inhibition is incompatible with long-term survival. Targeting the activity of this enzyme in colon cancer stem cells via BetA or chemical inhibitors could therefore be a therapeutic approach.

**Lipidomics**

In chapter 5, we describe the set-up of a lipidomics pipeline to improve the characterization and identification of phospholipids. To test this pipeline we used BetA treated samples. Using the pipeline we found that we could identify several clusters of peaks belonging to different phospholipids. On top of that we could identify also length and saturation grade of these phospholipids. This resulted in the identification of total phospholipid levels of vehicle and BetA treated samples. Differences in total phospholipid levels were mainly observed in phosphatidylcholine, phosphatidylglycerol and bis-(monoacylglycerol)-phosphate. However in these phospholipids no differences in the saturation grade was observed between samples. The only phospholipid that displayed a clear difference in saturation grade was cardiolipin. In BetA treated samples it was clearly visible that the side chains of cardiolipin are more saturated as compared to vehicle treated cells. This indicates that BetA specifically alters the saturation grade of cardiolipin which eventually results in cell death. With this pipeline we show that identification of phospholipids can be automated and this method can be used in the clinic for diagnostic purposes.

Finally, in chapter 6, the data described in this thesis are put in perspective. We showed that BetA-induced cell death does not result of necroptosis or autophagy but likely via a yet-undefined pathway. We discuss what the role of autophagy in BetA-induced cell death is and what the possible cell death mechanism can be. Moreover we show in this thesis that BetA-induced cell death evolves due to an inhibition of SCD-1 resulting in cardiolipin saturation and cell death. We discuss what role BetA, SCD-1 inhibition and cardiolipin have on cancer and cancer stem cells and how this can play a role in cancer therapy. In this chapter we give perspectives for further investigation to clarify the mechanism of BetA, as well as future interventions and therapies are proposed for both BetA and SCD-1 inhibitor. For the investigation of lipids we set-up a lipidomics pipeline and we give future perspectives of a lipidomics pipeline and use of this pipeline in the clinic.

In conclusion, we believe our data provide compelling evidence that lipid metabolism, specifically the saturation of cardiolipin, can serve as a reliable target in cancer therapy, which is effectively exploited by BetA.
“Well! I've often seen a cat without a grin,' thought Alice 'but a grin without a cat! It's the most curious thing I ever saw in my life!”

**Lewis Carroll, Alice in Wonderland**
Kanker is een veelvoorkomende ziekte met 100.000 nieuwe gevallen per jaar. De ziekte wordt gekenmerkt door ongecontroleerde groei van weefsels vanwege een aanhoudende celdeling. Gezonde cellen in het lichaam delen alleen wanneer dat nodig is (bijvoorbeeld om wondjes te genezen). Tijdens celdeling krijgen de cellen een specifieke functie en zodoende ook een specifieke vorm en grootte, dit proces heet differentiatie van de cel. Je lichaam heeft stamcellen die ervoor zorgen dat vanuit 1 cel een nieuwe groep cellen (die ook gedifferentieerd zijn) kan groeien. Het delen en differentiëren van cellen gebeurd door signalen die gegeven worden. Deze signalen kunnen van buiten de cel komen (zoals chemische stofjes, virussen) maar ook van binnenuit (eiwitten). Zowel de uitwendige als de inwendige signalen kunnen de cel laten delen of remmen de celdeling. Dit proces van groei stimulatie en remming van groei staat onder strenge controle in de cel. Als cellen ontsnappen aan deze controle en er een ontregeling plaatsvindt tussen de balans van groei stimulatie en groei remming in het voordeel van de groei factoren dan kan er een onbeheerste groei ontstaan. Deze onbeheerste groei kan goedaardig of kwaadaardig zijn. Bij kwaadaardige groei spreken we van kanker, deze cellen zijn in staat om het organa waarin ze zitten te vernietigen en kunnen zich ook verspreiden door het lichaam. Goedaardige tumorcellen beschadigen alleen het organa waarin ze zitten en kunnen zich niet verspreiden in het lichaam.

Tot op heden is kanker een ziekte die moeilijk te bestrijden is en zodoende wordt er veel onderzoek gedaan naar hoe kankercellen werken en hoe deze anders zijn ten opzichte van een gezonde cel. Momenteel zijn er vele soorten therapieën in de kliniek beschikbaar, echter zijn deze behandelingen (bv chemotherapie en radiotherapie) niet specifiek voor de kankercellen en werken ze ook op de gezonde cellen, waardoor de patiënt vaak last heeft van bijwerkingen. Door te ontdekken hoe een kankercel werkt, kan men gericht medicijnen zoeken die de kankercel dood maken, maar de gezonde cel geen schade berokkent. Daarnaast wordt er ook onderzoek gedaan naar nieuwe stoffen die chemisch gemaakt zijn of in de natuur zijn gevonden, deze nieuwe stoffen worden getest in een laboratorium om verschillende soorten kankercellen om te onderzoeken wat voor effect deze stoffen hebben op de vitaliteit van de cellen. Via zo’n soort test is Betulinezuur (BetA) ontdekt. Dit stofje dat voorkomt in onder andere berkenbomen blijkt in staat te zijn om kankercellen dood te maken terwijl het geen schadelijke effecten heeft op de gezonde cel. Dit proefschrift gaat over BetA en hoe het kankercellen dood maakt, daarnaast is er ook een hoofdstuk wat een bio-informatisch algoritme beschrijft over het ontdekken en benoemen van lipiden (vetten)

In hoofdstuk 1 beschrijven we de literatuur van de afgelopen jaren over kanker, de therapieën van kanker, de verschillende vormen van celdood die er zijn en het metabolisme (stofwisseling) van de cel en hoe deze anders is in een kankercel. Daarnaast wordt BetA geïntroduceerd, hoe het stofje ontlekt is, en wat er tot nu toe bekend is over onder andere het werkingsmechanisme van BetA geïnduceerde celdood.
Nederlandse Samenvatting

Het is bekend dat BetA een vorm van gereguleerde celdood induceert die apoptose wordt genoemd. Echter als we deze vorm van celdood remmen met specifieke remmers (caspase remmers) is er nog een groot deel van de kankercellen welke alsnog dood gaan. Dit is alleen mogelijk als er een ander celdood mechanisme is die dit veroorzaakt. In hoofdstuk 2 is er gekeken naar welke vormen van celdood er naast apoptose worden geïnduceerd door BetA. We zijn begonnen met het kijken naar necroptose, een vorm van gereguleerde celdood welke alleen kan optreden in een cel als er geen caspases zijn. Om te zien of necroptose deel uitmaakt van BetA geïnduceerde celdood hebben we naast apoptose ook necroptose geremd. De combinatie van deze remmers zouden als deze vormen van celdood door BetA geïnduceerd worden, moeten leiden tot 100% levende cellen aangezien we beide vormen van celdood geremd hebben. Echter zagen we in deze proef dat de kankercellen nog steeds dood gingen na behandeling met BetA, hieruit kunnen we concluderen dat een andere vorm van celdood betrokken moet zijn.

De aandacht is vervolgens gevestigd op autofagie, een vorm van celdood waarbij de cel zichzelf als het ware opeet om de opgegeten delen vrij te geven aan de omgeving om als voeding te dienen. Vaak wordt autofagie ook gezien als een overlevingsmechanisme, omdat de cel zichzelf opeet om andere cellen van bouwstenen te voorzien. Autofagie is een proces waarbij onderdelen van de cel in een blaasje worden gestopt, ook wel autofagosoosum (een soort vuilniszakje) genoemd. Om het “afval” op te ruimen moet het blaasje samensmelten met een lysosoom (de vuilverbranding), waarbij de inhoud van het blaasje wordt afgebroken en gerecycled. Dit proces van complete afbraak wordt ook wel autofagische flux genoemd.

In hoofdstuk 2 laten we zien dat BetA een massale vorm van autofagie induceert, door het meten van een eiwitvorm (LC3II) wat voorkomt op de blaasjes (vuilniszakken). Met behulp van de microscoop zagen we dikke groene puntjes van het eiwit ontstaan terwijl we met eiwitmetingen (Western Blot) zien dat de LC3II vorm zichtbaar wordt na behandeling met BetA. Soms gebeurt het wel eens dat het afval niet verwerkt wordt in de lysosomen en dat je daardoor zo’n hoge hoeveelheid LC3II meet. Om te zien of autofagische flux (de samensmelting van het blaasje met lysosoom en dus complete afbraak van het afval) plaatsvindt, is dit gemeten door gebruik te maken van een LC3 eiwit wat is gekoppeld aan een rode en groene kleur. Als rood en groen gemengd worden, ontstaat er een gele kleur, tijdens het proces van autofagische flux wordt de groene kleur afgebroken in het lysosoom terwijl de rode kleur blijft. Behandeling met BetA laat een rode kleur achter in de cellen wat tot de conclusie leidt dat er volledige afbraak is. Dit is ook aangetoond door het meten van afbraak van radioactieve lang levende eiwitten.

Mitochondriën zijn energiefabriekjes van de cel en ze zijn betrokken bij apoptose. Mitochondriën hebben poortjes die open en dicht kunnen, waarbij er stofjes van binnen het mitochondrion naar buiten kunnen (bv cytochrom c, wat celdood (apoptose) induceert).
Eerder onderzoek liet zien dat cyclosporine A (een stofje wat mitochondriale poortjes dicht houdt) de kankercellen in leven houdt door remming van apoptose. We waren benieuwd wat dit stofje doet op de autofagie inductie. We zagen dat er minder autofagie werd geïnduceerd in combinatie van BetA met cyclosporine A dan met BetA alleen. Dit suggereert dat geïnduceerde autofagie een gevolg is van de schade die BetA geeft aan de mitochondriën.

Bij autofagie eet de cel zichzelf op om een andere cel van voeding te voorzien. Autofagie is al bij lagere concentraties BetA te zien, mogelijk wijzend op dat autofagie vooral een overlevingsmechanisme is na BetA behandeling. Het is echter ook denkbaar dat de massale inductie van autofagie na BetA behandeling resulteert in een disbalans tussen overleving en celdood en dat autofagie inductie een celdood mechanisme is. Om dit te onderzoeken is er gebruik gemaakt van cellen die bepaalde eiwitten missen die belangrijk zijn voor autofagie en zodoende kunnen deze cellen kunnen niet langer autofagie-induceren. Als we deze cellen behandelden met BetA zagen we meer celdood ontstaan en dus is autofagie een overlevingsmechanisme in de cel. Het blijft echter onduidelijk welke vorm van celdood BetA naast apoptose induceert.

In hoofdstuk 3 bestudeerden we de vorm van apoptose meer in diepgang. Apoptose is een vorm van gereguleerde celdood die op 2 manieren plaats kan vinden. Via een receptor pathway (een sleutel en slot principe, je steekt een sleutel in een slot en het slot kan open, waarbij de sleutel een proces in gang zet waarbij stofjes aangezet worden die leiden tot celdood) of via een mitochondriale pathway (waarbij het energiefabriekje van de cel lek raakt en er stofjes (o.a. cytochrom c) vrij komen die celdood induceren). Omdat BetA de mitochondriale route induceert hebben we gekeken wat er gebeurt met de mitochondriën zelf. We zagen dat de structuur van de mitochondriën verandert na behandeling met BetA. Het binnen membraan (cristae) van de mitochondriën wat normaal gesproken lange strepen laat zien is na behandeling met BetA rond geworden. Dit is een zeer opmerkelijke waarneming en deze ronde structuren van de cristae van mitochondriën zijn ook zichtbaar in Barth syndroom patiënten. Deze patiënten hebben een defect in het Taffazin gen waardoor zij een meer verzadigde vorm hebben van cardiolipine (CL).

CL is een lipide (vet) wat vier vetzuurketens heeft en voornamelijk voorkomt in de mitochondriën waar het betrokken is bij de structuur van de cristae en tevens ook cytochrom c vasthoudt in de mitochondriën. De zijketens van CL kunnen verschillen in lengte en in verzadigingsgraad. Meer verzadigd vet (zoals roomboter) betekend dat deze minder flexibel is als onverzadigd vet (zoals olie). Het is belangrijk voor de cel om een juiste balans te hebben tussen verzadigd en onverzadigd vet, omdat veel verzadiging leidt tot starre vetten die niet meer flexibel zijn en aangezien vetten voorkomen in membranen kan dit leiden tot structuur veranderingen en verandering in functionaliteit van het membraan.
Omdat de veranderingen in de mitochondriale cristae na BetA behandeling zo lijken op de cristae in Barth syndroom patiënten hebben we de levels en verzadiging van CL gemeten. We zagen dat CL meer verzadigd raakt na behandeling met BetA. We vroegen ons toen af wat de oorzaak is van verzadiging. In het lichaam worden vetten opgenomen uit de voeding of kunnen worden aangemaakt. In kankercellen maakt de cel liever vetten aan dan dat het deze opneemt uit de voeding, waarschijnlijk omdat kankercellen zo snel delen en meer vetten nodig hebben als bouwsteent. Nieuwe vetten maken gebeurt via een ingewikkeld proces wat resulteert in een verzadigd vetzuur. Het lichaam maakt onverzadigde vetten via een enzym stearoyl-CoA desaturase 1 (SCD-1) wat het verzadigd vetzuur omzet in een onverzadigd vetzuur. Deze vetzuren kunnen dan in alle lipiden ingebouwd worden.

We bedachten dat BetA misschien invloed heeft op dit enzym, zodoende werd de activiteit van dit enzym gemeten. We zagen dat na BetA behandeling of behandeling met een SCD-1 remmer de activiteit van dit enzym was geremd en er dus meer verzadigd vetzuur aanwezig was in de cellen. De vraag was nu of remming van SCD-1 met een specifieke SCD-1 remmer ook dezelfde veranderingen in de mitochondriën opleverde, en inderdaad remmen van SCD-1 met de SCD-1 remmer leidt ook tot mitochondriale veranderingen en CL verzadiging. We hebben daarna gekeken of het toevoegen van extra onverzadigde vetzuren in combinatie met BetA de morfologie van de mitochondriën hersteld en of ook CL levels weer normaal werden. Het toevoegen van extra onverzadigde vetzuren bij BetA behandeling resulteerde in verminderdering van veranderingen in de mitochondriën en ook in CL verzadigingsgraad. Het tegenovergestelde werd aangetoond met de combinatie van verzadigd vetzuur en BetA, in deze combinatie werd de structuur van de mitochondriën nog slechter dan met BetA alleen en ook waren de CL levels meer verzadigd dan na BetA behandeling alleen. Daarnaast lieten we zien dat BetA of SCD-1 beiden cytochroom c vrij laten uit de mitochondriën en celdood induceren en dat de combinatie van SCD-1 remming (door BetA ofwel SCD-1 remmer) en extra verzadigd vetzuur tot meer uitscheiding van cytochroom c leiden en tot grotere inductie van celdood. Dit terwijl toevoeging van onverzadigd vetzuur de schadelijke effecten van BetA of SCD-1 tegengaan en er juist minder cytochroom c vrijgelaten wordt en er minder celdood ontstaat. In dit hoofdstuk hebben we een nieuwe celdood route ontdekt die specifiek kankercellen raakt. Gezonde cellen zijn minder afhankelijk van SCD-1 activiteit dan kankercellen en daardoor zijn kankercellen veel gevoeliger voor BetA.

Kankerstamcellen zijn een onderdeel van de tumor die zelf vernieuwende eigenschappen hebben en in verschillende andere cellen kunnen differentiëren. Er wordt van deze cellen gedacht dat zij zorgen voor het terugkomen van de kanker na behandeling en voor de verspreiding (metastasering) van de tumor door het lichaam. Kankerstamcellen kunnen namelijk van 1 losse cel een hele nieuwe groep kankercellen maken en kankerstamcellen zijn ook nog eens erg ongevoelig voor de verschillende vormen van therapie. Een grote uitdaging in het kankeronderzoek is dan ook het begrijpen hoe deze cellen werken en het
vinden van therapieën die ook deze cellen dood maken. In hoofdstuk 4 hebben we de werking van BetA op darmkankerstamcellen getest. Tot onze verbazing werkt BetA zeer effectief op kankerstamcellen en zelfs sneller dan in kankercellen. Al na 2 uur zien we een celdood inductie in de totale populatie van de cellen. Als we specifiek kijken naar het effect op kankerstamcellen zien we dat BetA ook in 10% van de kankerstamcellen celdood induceert en daarnaast werd ook een verlaging van zelfvernieuwing gevonden. De huidige standaardtherapie voor darmkanker, oxaliplatin bleek in langdurige experimenten minder effectief dan BetA in het verminderen van uitgroei van de darmkankerstamcellen. Dit suggereert dat bij oxaliplatin resistentie tegen het middel op lijkt te treden terwijl dit bij BetA niet gebeurd. Om te kijken of darmkankerstamcellen ook de beschreven nieuwe celdood route uit hoofdstuk 2 volgen werden darmkankerstamcellen behandeld met SCD-1 remmer. Ook met SCD-1 remmer werd een celdood geïnduceerd, echter was deze niet zo snel als bij BetA. Verder onderzoek naar het precieze mechanisme en de werking van BetA in darmkankerstamcellen is nodig.

Omdat het remmen van SCD-1 (via BetA of SCD-1 remmer) leidt tot een ophoping van verzadigde vetzuren die in elk lipide ingebouwd kunnen worden hebben we gekeken wat de effecten van BetA op lipiden in het algemeen is (dus niet alleen CL). Het meten van lipiden (vetten) is een langdurig en ingewikkeld proces. Zodoende hebben we in hoofdstuk 5 alle lipiden geanalyseerd met een nieuwe methode (lipide pipeline) die sneller en nauwkeuriger lipiden kan herkennen. We beschrijven de opzet van deze pipeline en testen we de werking van deze methode door gebruik te maken van BetA behandelde cellen. Met deze nieuwe bio-informatische methode kunnen we veel sneller en meer lipiden herkennen en benoemen dan voorheen. We hebben verschillende fosfolipiden kunnen identificeren en we hebben bekeken wat de verschillen waren tussen onbehandelde en behandelde cellen. We zagen dat er significante verschillen waren in de totale waarden van sommige lipiden (bv PG en BMP). In CL totaal levels is geen significant verschil gevonden terwijl we wel verschil zien in verzadigingsgraad van de zijketens. In PG en BMP daarentegen zien we verschillen in totale levels maar niet in de verzadiging. Waarom BetA alleen CL meer verzadigd maakt moet verder worden onderzocht.

In hoofdstuk 6 worden de resultaten van de voorgaande hoofdstukken bediscussieerd in de context van literatuur en wordt er besproken hoe BetA en SCD-1 remming een rol kunnen spelen in toekomstige therapie. Tevens bespreken we de rol van deze lipide pipeline in de kliniek.

Samenvattend laten we zien dat vetzuurmetabolisme, in het specifiek CL verzadiging, een zwakke schakel is in de kankercel en dat dit een mogelijk nieuw aangrijpingspunt is voor therapie.
List of Publications

“Why, sometimes I’ve believed as many as six impossible things before breakfast.”

Lewis Carroll, Alice in Wonderland
List of publications


