Mechanisms of Betulinic acid-induced cell death
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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.\(^{(3)}\) 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.\(^{(4)}\) 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.\(^{(5)}\) Apoptosis is a cell autonomous program of damaged or stressed cells
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. 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. 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. 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. The resulting complex, named ‘death-inducing signaling complex (DISC), constitutes a platform that regulates the activation of caspase-8 and caspase-10. Depending on cell type (type 1 or type 2 cells) two different routes of further downstream pathways are known. 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, which translates to the mitochondria and releases cytochrome c and results in mitochondrial outer membrane permeabilization (MOMP). 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. 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. 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. 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
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 sensitizer/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)
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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.

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**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. 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. 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. The phagophore expands so it can engulf cytosolic cargo (i.e organelles, ribosomes, protein aggregates) to form a double-membraned autophagosome. 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. 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. 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 )

The capacity for large scale degradation carries a certain risk for cells, as unregulated degradation of cytoplasm or organelles is likely to be lethal. 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.\(^{44}\)

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
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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.

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
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RIP3. The latter two proteins in turn form a multiprotein complex called the nescrosome, which further activates players for execution of necrotic cell death. 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, inhabitable by necrostatin 1 form of TNFR1 induced regulated necrosis), mitochondrial permeability transition (MPT)-dependent regulated necrosis (relies on cyclophilin D, reviewed in [62]), and parthanatos (a non-apoptotic cell death subroutine that critically relies on the (hyper)activation of poly(ADP-ribose) (PAR) polymerase 1 (PARP1). 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.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.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.67 Lysosome membrane permeabilization and the consequent leaking of hydrolases (mainly cathepsin proteases) into the cytosol, can initiate the intrinsic apoptosis pathway 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.66, 69, 70 This form of cell death where lysosomal hydrolases are the key activation step is called lysosomal cell death.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.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.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 in vitro and in vivo. (reviewed in 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-(monoacylglycero)-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.(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(88) and ovarian cancer.(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 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)} and 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.

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\textsuperscript{[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.\textsuperscript{[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.\textsuperscript{[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.\textsuperscript{[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.\textsuperscript{[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.
**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, aconitate 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; PDHK1, 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.\(^{(118, 119)}\) 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.\(^{(120-122)}\) Secondly, the Warburg effect leads to the
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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. 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.

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. 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. 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. Overexpression of mTOR results in an increase in surface expression of glucose transporters. The breakdown of lipids by β-oxidation is suppressed by PI3K/AKT. In hematopoietic cells PI3K/AKT suppresses carnitine palmitoyltransferase-1A (CPT1A). CPT1A is the rate-limiting enzyme for β-oxidation. To fulfill the need of unsaturated fatty acids SCD-1 is overexpressed in several tumors. These enzymes and genes mentioned above, have been proposed as novel targets for tumor treatment.

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. Lipids acts as signaling molecules in cancer, i.e. phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 is a produced by PI3K pathway in response to growth factor signaling and mediates the recruitment and activation of Akt. 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. 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. Taken together, it is clear that altered lipid metabolism has beneficial effects for tumors.
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 deacylase (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 cardio skeletal 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.
3 and is important for the apoptotic signal. 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.

**Figure 4: Cardiolipin synthesis and remodeling in eukaryotes.** In cardiolipin synthesis, phosphatidic 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. 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. 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.\(^{(179)}\) 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.\(^{(179)}\) 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\(^{(180)}\) 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.\(^{(181)}\) 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.\(^{(181)}\) 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.\(^{(182)}\) 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.\(^{(183)}\)

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.\(^{(4)}\) 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.\(^{(184)}\) However, the clinical responses to antiangiogenic drugs have been found to be transient.\(^{(185-187)}\) Clinical validation for this evasive resistance is shown by the increased invasion and local metastasis observed in glioblastomas treated with
antiangiogenic therapies.\textsuperscript{(188, 189)} 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.\textsuperscript{(4)}

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.\textsuperscript{(203, 204)} 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.\textsuperscript{(205)} 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 undrugable targets. On top of that, synthetic lethal therapeutic agents can be applied alone by monotherapy, or in combination with more conventional treatments.\textsuperscript{(206-208)} 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.\textsuperscript{(209, 210)}

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.\textsuperscript{(211)} 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.\textsuperscript{(211)} 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.\textsuperscript{(212)}

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, Selicilid</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 (Ritu) [19 194, 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 poliposis</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. 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. 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. CSCs are defined as a subset of tumor cells which possess self-renewal and multi-lineage differentiation potential. CSCs can be identified by various markers, and in vivo experiments have shown that CSCs very efficiently form tumors that resemble the original tumor from which the CSCs were derived. It has been suggested that CSCs are responsible for recurrences and metastasis, because they are thought to be therapy resistant (both chemo- and radiotherapy) and to effectively repopulate the tumor.

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. Vinca alkaloids were introduced into the clinics in late 1950s as Velban® and Oncovin®, and later on Eldisine® and Navelbine® followed. 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. Taxanes belong to the terpenoids and one well known compound is Taxol (from Taxus), which was shown in 1979 to stabilize microtubule assembly. It is used in the clinic for the treatment of various cancer types, including ovarian, lung and breast cancer. 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.
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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. 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. 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. Two years later, head and neck squamous cellular carcinoma cells were also shown to be sensitive to BetA 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. BetA was shown to be a very potent agent against tumor with EC50 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. 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. The efficacy of BetA was also shown in hepatoblastoma, rhabdomyosarcoma and nasopharyngeal carcinoma 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\textsuperscript{269} and in leukemia cells form patients and cell lines.\textsuperscript{270} The last study showed cell death inducing capacities of BetA independent of risk stratification, age and sex of the patient and leukemia type.\textsuperscript{270} BetA also induced cell death in human CML cell line K-562.\textsuperscript{271} 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 \textit{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.\textsuperscript{259} 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.\textsuperscript{272} Consistent with these data, BetA induced ROS production, which was inhibited by BCL-2 overexpression or antioxidants.\textsuperscript{262}

Although BCL-2 overexpression showed protection against BetA-induced apoptosis in SHEP and glioma cells, it only partially protected Jurkat cells\textsuperscript{270,273}, MCF-7 cells\textsuperscript{273} and melanoma cells\textsuperscript{274} 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.\textsuperscript{273} 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.\textsuperscript{273}

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.\textsuperscript{265} 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. 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. 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. BetA improved vincristine-induced cytotoxicity in mouse melanoma cells and reduced the number of metastases in vivo. 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. It was also shown that BetA sensitizes drug-resistant colon cancer cells and esophageal squamous carcinoma cells to oxaliplatin, irinotecan and 5-fluorouracil. The combination of BetA and docetaxel or 2-methoxyestradiol resulted in increased apoptosis. 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. 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. This combination also resulted in less discernible side effects than gemcitabine alone, which was used as a standard reference drug. 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.
Introduction

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. 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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