Mechanisms of Betulinic acid induced cell death
Potze, L.

Citation for published version (APA):

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
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.\(^8\)\(^9\) 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
cells. Cancer cells increase their *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 *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 *de novo* fatty acids for yet undefined reasons. The end product of *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.\(^{[10]}\) 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\(^{[11]}\) as normal cells do not utilize the *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).\(^{[12]}\) Unsaturated fatty acids are important precursors for various products in the cell, like phospholipids (cell membrane), diacylglycerols (signaling) and triglycerides (energy storage).\(^{[13]}\) Overexpression of SCD-1 is a result of the overall increase of *de novo* fatty acid synthesis where besides SCD-1 also sterol regulatory element binding proteins (SREBPs) are overexpressed.\(^{[14, 15]}\) 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 *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 *in vitro* and retards the growth of tumors *in vivo*.\(^{[16-20]}\)

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.\(^{[21, 22]}\) 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.\(^{29-32}\) 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.\(^{33}\)

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.\(^{34-36}\)

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.\(^{34}\) 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 \(^{34, 37}\) 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.\(^{38, 39}\)

Mitochondrial fission occurs at the same time as the activation of BAX, mitochondrial outer membrane permeabilization and the resulting cytochrome c release.\(^{40}\) On top of that, it was shown that disruption of OPA1 complexes results in cytochrome c release and mitochondrial cristae remodeling.\(^{23}\) 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\(^{34}\) 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 IIA 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 how 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 optimalization 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


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


Chapter 6
