Mechanisms of Betulinic acid-induced cell death

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Chapter 3

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

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

Lewis Carroll, Alice in Wonderland
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Abstract

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

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

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

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

Mitochondrial morphology is changed and CL saturation increased upon BetA treatment

In untreated cancer cells and normal cells, mitochondria appeared as a tubular network evenly divided throughout the cell (Figure 1a and Supplementary Figure 1). In contrast, in cancer cells treated with BetA the mitochondrial network changed in a concentration dependent manner and became more fragmented (Figure 1a and Supplementary Figure 1). Strikingly, this fragmentation is not observed in non-transformed human fibroblasts (Co18; Supplementary Figure 1). To gain more insight into these mitochondrial changes at the ultrastructural level, transmission electron microscopy was employed. Untreated cells contained mitochondria with a typical longitudinal cristae. Upon BetA treatment, mitochondria were more electron dense and displayed significant ultrastructural changes, in particular, concentric (circular shaped) cristae were evident (Figure 1b). Interestingly, such cristae abnormalities have previously been observed in tissues of Barth syndrome (BTHS) patients,\(^3\) a disease that is caused by a germline mutation in the Tafazzin gene, which encodes a transacylase involved in CL remodelling and maturation. Cells of BTHS patients display characteristic changes in CL, which cause mitochondrial ultrastructural as well as functional abnormalities.\(^3\)

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

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

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

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

Unsaturated FAs revert CL modification and mitochondrial changes

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

CL modification evokes cell death and decreases clonogenic survival

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

B

C

D

E

F

G

H

DMSO

BetA

PG(32x)

fold increase/decrease

BHTS vs. avg. control (2log)

Control BTHS

BHTS

fold increase/decrease

BHTS vs. avg. control (2log)
Figure 2. BetA-induced cardiolipin saturation (a) HeLa cells were treated for 18 h with vehicle (DMSO) followed by CL analysis by HPLC mass spectrometry. (b) HeLa cells were treated for 18 h with 10 μg/ml BetA followed by CL analysis by HPLC mass spectrometry. (c) Zoom in of CL C68 cluster (containing 2 x C16 and 2 x C18 fatty acids) of DMSO treated cells 18 h (d) Zoom in of CL C68 cluster of 10 μg/ml BetA treated cells. (e) Quantification of fold change of C68:X species in BetA treated (18 h) HeLa cells (n=8) compared to vehicle treated HeLa cells (n=8). (f) Zoom in of CL C68 cluster of control fibroblasts. (g) Zoom in of CL C68 cluster of BTHS patients. (h) Quantification of fold change of C68:X species in BTHS patients (n=3) compared to controls (n=3). HeLa cells were pre-treated for 4 hours with vehicle (DMSO) (i, j) or 50 μM heptadecanoic acid (C17:0) (k, l) after which vehicle (DMSO) (i, k) or 10 μg/ml BetA (j, l) was applied for 18 h. Zoom in of CL clusters of C68 and C69 (containing C17) are shown. (m) Quantitative analysis of CL content in C68 and C69 CL cluster of DMSO C17:0 treated cells (n=2). (n) Quantification of change of C68:X species in BetA treated HeLa cells compared to vehicle (DMSO) treated HeLa cells (n=2)
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### A

Saturated fatty acids

Unsaturated fatty acids

### B

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### D

#### DMSO

Relative intensity (%)

#### BetA

Relative intensity (%)

### E

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### G

- **DMSO**
- **SCD-1 Inhibitor**
BetA-induced cell death by cardiolipin saturation

Figure 3. Desaturase activity is instrumental in BetA-induced mitochondrial damage (a) SCD-1 enzyme, introduces a double bond in saturated fatty acids at the n-9 position producing unsaturated fatty acids. (b) HeLa cells were treated with vehicle (DMSO), 150 nM SCD-1 inhibitor or 10 µg/ml BetA for 18 h after which $^2$H$_2$-C16:0 was applied for additional 6 h. Conversion to $^2$H$_3$-C16:1 was measured. Mean ± SD of 3 experiments are shown. ***P<0.001. (c) HeLa cells were treated with vehicle, 150 nm SCD-1 inhibitor or 10 µg/ml BetA for 18 h after which $^2$H$_2$-C18:0 was applied for additional 6 h. Conversion to $^2$H$_3$-C18:1 was measured. Mean ± SD of 3 experiments are shown. ***P<0.001. (d) Zoom in of CL C68 clusters. HeLa cells were incubated with vehicle (DMSO), 10 µg/ml BetA, 50 µM POA, 50 µM PA or a combination of 10 µg/ml BetA with 50 µM POA or 50 µM PA for 18 h after which CL analysis was performed using HPLC mass spectrometry (e) Quantification of CL saturation as measured in (d). Relative C68 CL saturation is shown which is calculated as (C68:2 + C68:3) / (C68:4 + C68:5). Mean ± SD of 2 experiments are shown. * P< 0.05; ns, not significant. (f) HeLa cells were subjected to vehicle (DMSO) or 200 nM SCD-1 inhibitor with or without 50 µM POA or 50 µM PA for 40 h after which CLs were analysed by HPLC mass spectrometry. Relative C68 CL saturation is shown which was calculated as (C68:2 + C68:3) / (C68:4 + C68:5). Mean ± SD of 3 experiments are shown. **P< 0.01; ***P<0.001. (g) HeLa cells were treated with vehicle (DMSO) or 200 nM SCD-1 inhibitor for 40h after which cells were analysed via TEM. (h) HeLa cells with MitorRFP were subjected to vehicle (DMSO) or 10 µg/ml BetA with or without 50 µM POA or 50 µM PA and after 18 h cells were analysed via confocal microscopy. Pictures representative for 3 experiments are shown. (i) HeLa cells were subjected to vehicle (DMSO), 10 µg/ml BetA with or without 50 µM POA or 50 µM PA and after 18 h cells were analysed via TEM. Pictures representative for 3 experiments are shown.
such increases the incorporation of toxic saturated FA into lipids. Sustaining a stable pool of unsaturated FAs, by exogenous addition, therefore maintains the unsaturation grade of CL, mitochondrial integrity and thereby allows for cell survival. The maintenance of the unsaturated FA pool exerted long-term protection as it even resulted in the recurrence of clonogenic potential of BetA or SCD-1 inhibitor-treated cells. Single addition of these compounds resulted in a complete loss of clonogenic growth (Figure 4g and h), which was regained by the simple addition of POA. This effect was selective for this pathway and for instance not observed when cells were treated with classical chemotherapy such as etoposide (Supplementary Figure 8D). Taken together these data indicate that tumour cells carry an intrinsic mitochondrial vulnerability that can be effectively targeted by inhibition of SCD-1, using either specific inhibitors or the natural compound BetA and is not observed in normal fibroblasts. This vulnerability depends on enhancing the saturation of CL leading to altered mitochondrial structure, membrane permeability and as a consequence cell death and loss of clonogenic growth occur.

Discussion

Altered lipid metabolism is a key feature of tumour cells and required to sustain the constant need for new biomass. Our current study provides strong evidence for an unexpected mitochondrial vulnerability of tumour cells that depends on this de novo lipid synthesis. De novo FA synthesis generates intrinsically toxic FAs, which need to be converted by SCD-1 into monounsaturated FAs. Here we show that inhibition of this final step in the generation of preferred FAs results in cell death and loss of clonogenic growth. Interestingly, we confirmed that normal cells do not contain high levels of SCD-1 expression or activity consistent with the idea that these cells do not utilize de novo FA synthesis but instead acquire unsaturated FA from the surrounding. Although this effect of SCD-1 inhibition on tumour cells has been documented before, our data now provides an unexpected mechanistic explanation of this observed toxicity. Firstly, we show that SCD-1 inhibition quickly impacts on the composition of CL FA side chains, resulting in mitochondrial abnormalities, cytochrome c release and subsequent cell death. Secondly, we demonstrate that BetA, which is a potent anti-cancer agent, does so by inhibiting SCD-1 and therefore utilizes this tumour cell vulnerability. Together these data point to an upstream effect of BetA on CL biosynthesis. The fact that SCD-1 inhibitors mimic the effect of BetA and the observation that both can be reverted by the simple addition of unsaturated FAs all point to SCD-1 as the vulnerability in the CL biosynthesis pathway of tumour cells. Previously, we observed that BetA-induced cell death is tumour selective and that normal cells are not affected at doses that kill tumour cells both in vitro and in vivo. In agreement, the BetA or SCD-1-induced mitochondrial fragmentation in cancer cells is not detected in normal cells. The here proposed mechanism of action could
Figure 4. CL modification evokes cell death and decreases clonogenic survival. (a) HeLa cells were pretreated for 24h with 50 µM POA after which vehicle (DMSO) or 10 µg/ml BetA was applied for 40h. Other samples were left untreated for 24h after which 50 µM PA with or without 10 µg/ml BetA for 40h was applied. After treatment cytochrome c release was assessed via FACS. Data of 3 independent experiments are shown. * P<0.05, ns not significant. (b, c) HeLa cells were pre-treated with 50 µM POA after which vehicle (DMSO) or 10 µg/ml BetA was applied for 48h. Other samples were left untreated for 24h after which 50 µM PA with or without 10 µg/ml BetA for 48h was applied. Cell death was assessed via PI exclusion. Mean ± SD of 3 experiments are shown. * P<0.05, ns not significant. (d) HeLa cells were subjected to vehicle or 75 nM SCD-1 inhibitor with or without 50 µM POA or 50 µM PA for 72h after which cell death was assessed via PI exclusion. Mean ± SD of 3 experiments are shown. ** P<0.01; *** P<0.001. (e) HeLa cells were treated for 48h with vehicle (DMSO) or 10 µg/ml BetA with or without 50 µM POA or 50 µM PA after which fresh medium was applied for 72h. Cells were stained with crystal violet blue for 6h. Mean ± SD of 3 experiments are shown. ** P<0.01. (f) HeLa cells were treated for 72h with 75 nM SCD-1 inhibitor with or without 50 µM POA or 50 µM PA after which fresh medium was applied for 48h. Cells were stained with crystal violet blue for 6h. Mean ± SD of 2 experiments are shown. *** P<0.001.
provide an explanation to this tumour selectivity. First, tumour cells appear addicted to de novo FA synthesis\textsuperscript{2–5} and as such continuously generate saturated FAs that need to be converted to unsaturated FAs. It is not completely clear why tumour cells prefer this synthetic pathway even in the presence of exogenous FAs. Except for adipocytes, this pathway is not utilized by normal cells, which acquire their FAs from exogenous sources.\textsuperscript{38} The latter provides a stable source of unsaturated FAs, and as such alleviates the need for desaturase activity in normal cells. Second, tumour cells have previously been shown to already contain CL species that are relatively more saturated as compared with normal cells.\textsuperscript{39} This could be a consequence of the apparently higher turnover of CL in tumour cells allowing for a less intense side chain modification or, alternatively, may be caused by the fact that mainly monounsaturated FAs are generated by de novo synthesis. Indeed, saturated FAs are abundantly found in cancer cells and also an increased content of monounsaturated at the expense of poly-unsaturated FAs is observed in transformed and cancer cells.\textsuperscript{40–42}

The higher saturation of CL suggests that tumour cells are closer to an unacceptable level of saturation than normal cells and therefore appear to be nearer to the tipping point of detrimental mitochondrial ultrastructural modifications and subsequent mitochondrial permeability. In agreement, BTHS patients contain a similarly low level of unsaturated FA side chains in CL and contain the same ultrastructural changes. However, this modification by itself is not sufficient for the actual mitochondrial leakage to occur as most cells in BTHS patients do not undergo cell death, suggesting that additional changes are needed. These could involve increased ROS production, which is often observed in tumour cells\textsuperscript{43,44} or a change in the way mitochondria are utilized in tumour cells, in particular in relation to the so-called Warburg effect.\textsuperscript{45,46} In agreement, our data show that BetA exerts cell death more rapidly and more vigorously than SCD-1 inhibition, which supports the idea that BetA is more potent in inducing cell death despite the fact that we observe more potent SCD-1 activity inhibition with SCD-1 inhibitors. We believe this is due to the fact that BetA enhances the need for new FA production. In essence we think that BetA not only stops the production of unsaturated FA, but on the other end increases the turnover of lipids and thereby the incorporation of saturated FA, which in the case of CL results in the toxic effects on mitochondria. This could explain the tumour selectivity as normal cells are not as dependent on SCD-1 and can maintain a relatively stable pool of unsaturated FA by uptake from the environment. The nature of this enhanced lipid turnover is not completely clear, but could for instance be a result of the massive induction of autophagy observed with BetA,\textsuperscript{67} which generates an enormous need for de novo membrane formation and thus lipid synthesis. Further insight into these additional changes may reveal another vulnerability that converges on the same pathway. Clues for these changes may come from neutrophils in BTHS patients as these display a relatively high level of apoptosis and indeed these patients frequently suffer from neutropenia.\textsuperscript{32,47}
The direct link between BetA-induced cell death and mitochondria is consistent with previous data which indicate that cell death ensues in a BAX and BAK-independent manner, but requires mitochondria and opening of the PT-pore. In agreement, cyclosporine A, an inhibitor of the PT-pore, blocks cell death induced by BetA and data not shown. Interestingly, recent data suggested that the Fo c-subunit ring of F1Fo ATP synthase can form a channel, which can lead to uncontrolled depolarisation and MOMP when opened persistently. Whether this represents the PT-pore remains to be confirmed, but it is important to mention that F1Fo ATP synthase as well as several of the other proposed constituents of the PT-pore associate with CL and CL can regulate PT-pore opening. Similarly, cytochrome c, which is released upon BetA treatment, interacts directly with CL through two independent binding sites. Moreover, Kagan et al. found that CL-bound cytochrome c acts as a peroxidase capable of catalysing H2O2-dependent peroxidation of CL and that this CL oxidation is an essential step in the release of cytochrome c during apoptosis. Whether the increased CL saturation has similar effects on the release of cytochrome c needs to be determined, but is a reasonable possibility. However, this initial release of cytochrome c from the inner membrane is not sufficient for apoptosis to occur. This requires mitochondrial outer membrane permeabilization either through BAX-dependent pore formation or through opening of the PT-pore. As BetA-induced death depends on the latter it seems likely to assume that CL saturation also affects the opening of the PT-pore.

In addition to the release of cytochrome c, mitochondrial fragmentation was rapidly observed in cells that have impaired SCD-1 activity and therefore modified CL FA side chains. The mitochondrial network is maintained by a continuous process of fusion (elongation of mitochondria) and fission (fragmentation of mitochondria). It is debated whether fusion/fission is important for cytochrome c release, but during apoptosis mitochondria are remodelled via activation of the fission machinery and synchronal neutralization of the fusion machinery. Importantly, this favoured mitochondrial fission occurs at the same time as BAX activation and permeabilization of the outer mitochondrial membrane, leading to cytochrome c release, suggesting that the events could be coupled. Mitochondrial fusion/fission itself is orchestrated by proteins such as Drp1 and Opa1, but also the structural, flexible, properties of CL. The observed mitochondrial fragmentation could therefore also be a direct effect of increased CL saturation, but how this would favour fragmentation is unclear and will require further analysis. Combined, our data point to a rapid effect of SCD-1 inhibition on the structural flexibility of CL and subsequently the mitochondrial integrity. Previous observations highlighted the importance of SCD-1 in tumours. Neoplastic cells contain higher levels of SCD-1 as compared with normal human skin fibroblasts and SCD-1 expression and activity are increased in several types of cancer. Moreover, normal cells appear to be resistant to the cytotoxic effect of SCD-1 inhibition. These observations suggests that a constantly active SCD-1 must be present in
cancer cells in order to maintain the level of unsaturated FAs to be incorporated in major cell lipids and thereby protect the tumour cells from excess saturated FAs.\textsuperscript{13,15,59–62} In agreement, knockdown of SCD-1 or inhibition with a specific small molecule inhibitor led to impaired biosynthesis of FAs, cholesteryl ester, triacylglycerol and phospholipid synthesis\textsuperscript{12,15,63,64} and induced death, confirming a pivotal role for SCD-1 in cancer.\textsuperscript{63} Several studies have reported the use of pharmacologic inhibitors of SCD-1 to reduce tumour growth in preclinical cancer models.\textsuperscript{65,66} In line with our data, toxicity induced by impaired SCD-1 expression sensitized cancer cells to the pro-apoptotic effects of PA.\textsuperscript{12,64} Our data now provide mechanistic insight and indicate that inhibition of SCD-1 rapidly changes the side chain saturation of CL and impairs normal mitochondrial structure. We conclude that CL is a crucial player in mitochondrial integrity that is strongly dependent on the level of saturation of its side chains. Targeting of this saturation or potentially even CL synthase in cancer cells provides a tumour-selective cell death mechanism that is induced by BetA.

\section*{MATERIALS AND METHODS}

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

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

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

**RNA isolation and quantitative PCR**
RNA was isolated using the Superscript III Reverse Transcriptase Kit (Invitrogen, Waltham, MA, USA). Total RNA was retrotranscribed using Nucleospin RNA Kit (Macherey-Nagel, Düren, Germany). Quantitative PCR analysis was performed using the following primers: SCD-1-F (5’-TCTGCTCCTCCTTCC-3’), SCD-1-R (5’-TGGACTCACCAGTCAC-3’), GAPDH-F (5’-AATCCATCACCACATG-3’) and GAPDH-R (5’-TGGACCTCAGACGTACTCA-3’).

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

**Electron microscope**
After the indicated treatment, cells were fixed in 1% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (McDowell fixative) and postfixed with 1% osmiumtetroxide (OsO4, Electron microscopy sciences, Hatfield, PA, USA) and kaliumhexacyanoferrate (K3FE(CN)6), VWR, Amsterdam, The Netherlands) in cacodylate buffer. Subsequently, the cells were dehydrated in an alcohol series and embedded into Epon (LX-112 resin Ladd Research, Williston, VT, USA). Ultrathin sections were collected on formvar-coated grids, counterstained with uranil acetate and lead citrate and visualized with transmission electron microscope (FEI technai 12).

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

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

**Desaturase activity measurements**
Cells were treated for 18 h with 10 μg/ml BetA or 150 nM SCD-1 inhibitor after which 50 μM \(^2\)H\(^3\)-palmitate (\(^2\)H\(^3\)-C\(^{16}\)O; Cambridge Isotope Laboratories) or for HeLa cells, 50 μM \(^2\)H\(^5\)-stearate (\(^2\)H\(^5\)-C\(^{18}\)O; Cambridge Isotope Laboratories) was added for an additional 6 h. Conversion of these saturated deuterium-containing FAs to unsaturated \(^2\)H\(^3\)-C\(^{16}\):1 or \(^2\)H\(^5\)-C\(^{18}\):1 was measured as described before.69,70

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

**Statistical analysis**
Statistical analyses were performed with Prism 5 (GraphPad software, La Jolla, CA, USA) applying a 2-tailed unpaired Student t-test. Differences were considered significant when P-values, *P<0.05; **P<0.01; ***P<0.001.
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Conflict of interest

The authors declare no conflict of interest.

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Chapter 3

References


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Supplementary figures

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

**Supplementary Figure 2: Induction of CL saturation upon BetA treatment.** MCF-7 cells were subjected to vehicle (DMSO) or 10 µg/ml BetA for 24 h after which CL content was measured. Relative C68 CL saturation is shown, which is calculated as (C68:2 + C68:3) / (C68:4). Mean ± SD of 2 experiments are shown. * P< 0.05; ns, not significant. (B) MCF-7 cells were subjected to vehicle (DMSO) or 10 µg/ml BetA for 24 h after which CL content was measured. Quantification of CL C68 cluster is shown. Mean ± SD of 2 experiments are shown.
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Supplementary Figure 3: Heptadecanoic acid incorporates into CL. HeLa cells were pre-treated with vehicle (DMSO) or 50 µM heptadecanoic acid for 4 h after which vehicle (DMSO) (A) or 10 µg/ml BetA (B) was applied for 18 h followed by CL analysis by HPLC mass spectrometry. Note the higher abundance of the C17 containing PG (33:0) compared to that of PG (33:1) in BetA treated cells.

Supplementary Figure 4: Desaturase activity and levels in cancer cells. (A) Indicated cell lines were analysed for their relative SCD-1 mRNA levels by qPCR. (B) HeLa cells with SCD-1 shRNA were treated with doxycycline for 72 h to induce knockdown of SCD-1. Levels of SCD-1 mRNA levels were measured by qPCR. (C) Indicated cell lines were treated with vehicle (DMSO), 150 nM SCD-1 inhibitor or 10 µg/ml BetA for 18 h after which 2H3-C16:0 was applied for additional 6 h. Conversion to 2H3-C16:1 was measured. Mean ± SD of 3 experiments are shown. ***P<0.001, **P<0.01.
Supplementary Figure 5: SCD-1 inhibitor induced mitochondrial morphology changes. HeLa cells with MitoRFP were subjected to vehicle (DMSO), 200 nM SCD-1 inhibitor with or without 50 μM POA or 50 μM PA for 40 h after which cells were analysed by confocal microscopy. Representative pictures of 2 experiments are shown.
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Supplementary Figure 6: CL levels of BetA and SCD-1 inhibitor treated cells (A) HeLa cells were subjected to vehicle (DMSO) or 10 μg/ml BetA with or without 50 μM POA or 50 μM PA for 18 h after which CL content was measured. Quantification of CL C68 cluster is shown. Mean ± SD of 5 experiments are shown (B) A549 cells were subjected to vehicle (DMSO) or 10 μg/ml BetA with or without 50 μM POA or 50 μM PA for 18 h after which CL content was measured. Relative C68 CL saturation of A549 cells is shown, which is calculated as (C68:2 + C68:3) / (C68:4 + C68:5). Mean ± SD of 3 experiments are shown. * P< 0.05; ns, not significant. (C) A549 cells were subjected to vehicle (DMSO) or 10 μg/ml BetA with or without 50 μM POA or 50 μM PA for 18 h after which CL content was measured. Quantification of CL C68 cluster is shown. Mean ± SD of 2 experiments are shown. (D) HeLa cells were subjected to vehicle (DMSO) or 200 nM SCD-1 inhibitor with or without 50 μM POA or 50 μM PA for 40 h after which CL content was measured. Quantification of the CL C68 cluster is shown. Mean ± SD of 3 experiments are shown.
Supplementary Figure 7: Effect of POA and PA on mitochondria (A) HeLa cells with MitoRFP were subjected to vehicle (DMSO), 50 µM POA or 50 µM PA for 18h after which cells were analysed via confocal microscopy. Pictures of 3 individual experiments are shown. (B) HeLa cells were treated with 50 µM POA or 50 µM PA for 18h after which cells were analysed via TEM.
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Supplementary Figure 8: CL modification evokes cell death and decreases clonogenic survival (A) HeLa cells with induced knockdown of SCD-1 D6 and H12 were left for 5 days, after which cell death was assessed via PI exclusion. (B) HeLa cells were treated with indicated concentrations of SCD-1 inhibitor for 96 h after which cell death was assessed via PI exclusion. (C) HeLa cells were treated with vehicle (DMSO), 10 μg/ml BetA, 75nM SCD-1 inhibitor (+), 200 nM SCD-1 inhibitor (++) or combinations of BetA and SCD-1 inhibitor for 96h after which cell death was assessed via PI exclusion. (D) HeLa cells were treated for 48 h with vehicle (DMSO) or 1 μg/ml etoposide with or without 50 μM POA or 50 μM PA after which fresh medium was applied for 72 h. Cells were stained with crystal violet blue for 6 h. Mean ± SD of 3 experiments are shown.