Targeting the Metabolic Response to Statin-Mediated Oxidative Stress Produces a Synergistic Antitumor Response

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ABSTRACT

Statin inhibitors are widely prescribed inhibitors of the mevalonate pathway, acting to lower systemic cholesterol levels. The mevalonate pathway is critical for tumorigenesis and is frequently upregulated in cancer. Nonetheless, reported effects of statins on tumor progression are ambiguous, making it unclear whether statins, alone or in combination, can be used for chemotherapy. Here, using advanced mass spectrometry and isotope tracing, we showed that statins only modestly affected cancer cholesterol homeostasis. Instead, they significantly reduced synthesis and levels of another downstream product, the mitochondrial electron carrier coenzyme Q, both in cultured cancer cells and tumors. This compromised oxidative phosphorylation, causing severe oxidative stress. To compensate, cancer cells upregulated antioxidant metabolic pathways, including reductive carboxylation, protein synthesis, and cystine import. Targeting cystine import with an xCT transporter—lowering MEK inhibitor, in combination with statins, caused profound tumor cell death. Thus, statin-induced ROS production in cancer cells can be exploited in a combinatorial regimen.

Significance: Cancer cells induce specific metabolic pathways to alleviate the increased oxidative stress caused by statin treatment, and targeting one of these pathways synergizes with statins to produce a robust antitumor response.

See related commentary by Cordes and Metallo, p. 151

Introduction

The mevalonate pathway plays an important role in cellular and systemic physiology, with downstream pathways contributing to the proper functioning of a diverse set of biological processes. Synthesis of mevalonate occurs by concatenating 3 acetyl-CoA molecules, followed by a reduction step by the enzyme HMG-CoA reductase. Mevalonate is then subject to further phosphorylation and decarboxylation steps, together catalyzing the production of 5-carbon isoprenoid molecules, three of which are used to synthesize a 15-carbon farnesyl pyrophosphate (FPP) molecule (Fig. 1A). Isoprenoids and FPP feed into separate downstream pathways to produce a variety of biomolecules. These are (i) dolichols, which are made up of varying numbers of isoprene units and act as anchors for glycosylation structures, (ii) prenyl units for protein prenylation, enabling their anchoring into membranes (1), (iii) coenzyme Q (CoQ), or ubiquinone, an essential electron carrier in the electron transport chain (2), and (iv) cholesterol. Cholesterol is arguably the best studied product of the mevalonate pathway, and is an important lipid that acts as a structural component of mammalian cell membranes and a precursor for the synthesis of steroid hormones, bile acid, and vitamin D (3).

Although cholesterol contributes to a variety of important physiologic processes, excess levels (hypercholesterolemia) are often observed in individuals with metabolic syndrome, and it is strongly linked to cardiovascular disease (4). This has spurred significant interest into potential pharmacologic interventions, leading to the development of statins. These are a class of drugs that inhibit the rate-limiting enzyme producing mevalonate, HMG-CoA reductase (HMGCR), and are thought to exert their beneficial effect by normalizing systemic cholesterol levels. Because of their favorable efficacy and safety profiles, statins are now among the most widely prescribed medicines in the clinic (5).

In recent years there have been many reports linking upregulated activity of the mevalonate and downstream metabolic pathways to cancer development and progression (6, 7). For example, in breast cancer, it was found that high HMGCR and additional mevalonate pathway gene transcript levels correlated with poor prognosis (6). Likewise, comparison of pancreatic ductal adenocarcinoma (PDAC) and normal pancreas tissue revealed strongly deregulated cholesterol metabolism (8). In addition, it was recently shown that p53 represses the mevalonate pathway and this is one mechanism by which it suppresses tumor development (9). Finally, a commonly occurring driver oncogene, Ras, is prenylated to facilitate its localization to the inside of the plasma membrane. This has been shown to cooperate with increased HMGCR levels to promote transformation (6).
Despite the clear dysregulation of the mevalonate pathway in a variety of cancer types, robust evidence for a therapeutic benefit to statin treatment remains limited to only a subset of cancer types. Statin use in combination with androgen deprivation therapy increased time to progression in prostate cancer (10). Similarly, in breast cancer, statin use postdiagnosis led to a reduced risk of recurrence, although the largest increase was observed when statin use was combined with angiotensin receptor blocker treatment (11).
Notably, in the subset of cancers where statins do appear to be effective, this cannot merely be attributed to their effect on protein prenylation (12), indicating that other parts of the mevalonate and downstream pathways contribute to tumorigenesis and could be blocked to achieve clinical benefit.

This discrepancy between upregulated activity of the mevalonate and branching pathways, and the limited clinical effect of statins in most cancer types, suggested to us that cells may be able to adapt their metabolism in response to statin treatment. Here, we performed a comprehensive stable isotope tracing study with both 13C and 2H tracers, to determine which branches of mevalonate metabolism are active in cancer cells and tumors, and how they may be affected by statin treatment. This led to the finding that biosynthesis of coenzyme Q (CoQ) was very pronounced in cultured cells and in tumors. CoQ depletion due to statin treatment may be affected by statin treatment. This led to the finding that biosynthesis of coenzyme Q (CoQ) was very pronounced in cultured cells and in tumors. CoQ depletion due to statin treatment.

Materials and Methods

Cell culture

KPC mouse cells originally derived from Pdx1-Cre; KrasG12D;+/T(172)/C (KPC) mice (C57BL/6j background), were kindly provided by Dr Jennifer Morton (Cancer Research UK Beatson Institute, UK). MIA PaCa-2 PDAC cells were purchased from the ATCC. The PC3 prostate cancer cell line was kindly provided by Dr Hing Leung (Cancer Research UK Beatson Institute, UK). All cells were routinely passaged in DMEM (Sigma) with 25 mmol/L glucose and 2 mmol/L L-glutamine, supplemented with 5% (v/v) serum (FBS; Sigma; base medium). Cells were split at 80% confluence every 6 weeks using a luciferase-based Mycoalert Mycoplasma Analyzer. Cells were plated for experiments two passages after thawing.

For media extracts, samples were centrifuged at 16,100 g for 5 minutes at 4°C before being scraped into high-performance liquid chromatography (HPLC) vials and 20 µL of lathosterol (100 ng/µL) internal standard added.

Cholesterol extraction and derivatization from cells, medium, and tissues

For cellular extracts, at the time of extraction, cells were first washed three times with 1 mL 4°C PBS before 700 µL 4°C extraction buffer (1.9 v/v water: methanol) was added. Plates were incubated for 5 minutes at 4°C before being scraped into high-performance liquid chromatography (HPLC) vials and 20 µL of lathosterol (100 ng/µL) internal standard added.

For media extracts, samples were centrifuged at 16,100 x g for 5 minutes at 4°C to remove cell debris and 500 µL supernatant vortexed.
for 15 minutes at 3,000 rpm at 4°C with 500 μL 1:1 v/v chloroform: methanol and 20 μL of lathosterol (100 ng/μL, Sigma) internal standard. Samples were centrifuged at 16,100 × g for 5 minutes at 4°C, the bottom chloroform layer extracted, transferred to an HPLC vial, and dried under N₂. Samples were resuspended in 750 μL cold extraction buffer (1:9 v/v water:methanol). For tissue, 700 μL 4°C extraction buffer and 20 μL of lathosterol (as an internal standard) was used as detailed in tissue extraction.

All samples (cell extracts, medium, tissue) were saponified by heating for 60 minutes at 80°C with 75 μL of 10 mol/L NaOH to obtain the total cholesterol pool. Upon cooling to room temperature, 200 μL water was added, followed by 500 μL n-hexane. Vials were vortexed for 5 minutes at 3,000 rpm, and the upper hexane layer transferred to an autosampler vial. The n-hexane extraction was repeated and samples dried under N₂. Samples were reconstituted in 50 μL dry pyridine and 50 μL N-Methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA, Sigma) silylation agent added. Samples were heated at 60°C for 60 minutes before cooling and immediate analysis by gas chromatography–mass spectrometry (GC-MS).

**Dolichol and CoQ extraction**

At time of extraction, cells were placed on ice and washed three times in 1 mL cold PBS before 750 μL extraction buffer (1:1 v/v PBS: methanol) was added to cells and scraped into 1.5 mL Eppendorf tubes and 500 μL chloroform added. Similarly, for tissues following homogenization as detailed above, 750 μL extraction buffer and then 500 μL chloroform added was added to sample. For all samples, 50 μL 1 mg/mL methanolic butylated hydroxytoluene (BHT, Sigma); SPLASH lipidomix internal standard mix (Avanti Polar Lipids) at 1 μL per 5 mg tissue/10⁵ cells and 7H₄-CoQ₁₀ (Sigma) at 500 ng/μL per 5 mg tissue/10⁵ cells was added. Samples were centrifuged at 10,000 g for 5 minutes at 4°C. Tubes were centrifuged at 16,100 g for 5 minutes at 4°C and the supernatants were transferred into HPLC vials. Vials were stored at −80°C prior to LC-MS analysis.

**Metabolite extraction**

Intracellular metabolite extraction was performed as follows: on ice, cells were washed three times in 1 mL cold PBS before 500 μL (4°C) extraction buffer (methanol, acetonitrile, and water, (5:3:2) v/v) was added. After 5 minutes, cells were scraped into Eppendorf tubes and shaken for 15 minutes at 3,000 rpm at 4°C. Tubes were centrifuged at 16,100 × g for 5 minutes at 4°C and the supernatants were transferred into HPLC vials. Vials were stored at −80°C prior to LC-MS analysis.

Media samples were centrifuged at 16,100 × g for 5 minutes at 4°C to remove cell debris and the supernatant vortexed for 15 minutes at 3,000 rpm at 4°C with cold extraction buffer (methanol, acetonitrile, and water, 50:30:20 v/v). Samples were centrifuged at 16,100 × g for 5 minutes at 4°C, and the supernatants were transferred into HPLC vials. In addition, a pooled sample of supernatants was created. A series of standard curves were created with pooled samples spiked with increasing concentrations of [U-¹³C]-labeled glucose, lactate, glutamate, and glutamine, which were used to determine the concentration of glucose, lactate, glutamate, and glutamine in media samples. For cystine, peak area was used for a relative concentration to be calculated.

Tissues were reconstituted in extraction buffer (methanol, acetonitrile, and water, 50:30:20 v/v) at 5 mg/mL tissue.

**Acetone analysis by GC-MS**

Acetone was analyzed using an Agilent 7890B GC system coupled to a 7890B Agilent Triple Quadrupole GC-MS system, with a Phenomenex ZB-1701 column (30 mm × 0.25 mm × 0.25 μm). An initial temperature of 40°C was set to increase at 10°C/minute up to 100°C, and held for 0 minutes. The instrument was operated in split mode (220:1) in the electron impact mode, 70eV.

**Cholesterol analysis by GC-MS**

Cholesterol was analyzed using an Agilent 7890B GC system coupled to an Agilent 7000 Triple Quadrupole GC-MS system, which was operating in a single quadrupole mode, with a Phenomenex ZB-1701 column (30 mm × 0.25 mm × 0.25 μm). An initial temperature of 200°C was set to increase at 20°C/minute up to 280°C, and held for 9 minutes. The instrument was operated in splitless mode in the electron impact mode, 70eV, for quantification and 50eV for labeling experiments. Cholesterol was quantified and isotope labeling pattern analyzed using Mass Hunter B.06.00 software (Agilent). Cholesterol and lathosterol internal standard peak areas were extracted from mass-to-charge ratio (m/z) 458 for both. Cholesterol was normalized to the internal standard, and a standard curve was used to quantify mg cholesterol per sample.

**CoQ and dolichol analysis by LC-MS**

LC-MS analysis was performed as described in ref. 17. CoQ was analyzed in positive mode using spray voltage 3 kV. Full MS (scan range 300–1,600 m/z) was used at 70,000 resolution with 10⁶ automatic gain control and a maximum injection time of 250 ms. For CoQ quantification, XCalibur Software (Thermo Fisher Scientific) was used to analyze peak height of CoQ₉, CoQ₁₀, and ⁷H₂-CoQ₁₀ internal standard. Similarly, for dolichols, peak height of dolichol-19 in dolichol internal standard mix (Avanti) was analyzed. Peak heights were normalized against both the internal standard and cell number.

**Metabolite analysis by LC-MS**

This was performed as described in ref. 18. Peak areas were determined using Thermo TraceFinder software. Metabolites were identified by a combination of exact masses of ions and retention times. This was validated using commercial standards of all detected metabolites run on the system prior to analysis. Peak areas were normalized to cell number.

**Body water ²H₂O enrichment calculations**

Acetone was quantified and isotope labeling pattern analyzed using Mass Hunter B.06.00 software (Agilent). Mass isotopologs 58 and 59 were integrated and their ratio compared with a standard curve to quantify plasma ²H₂O enrichment, as described previously (15, 16).

**Cholesterol, CoQ, and dolichol ¹³C and ²H tracing calculations**

For both [U-¹³C]-glucose/glutamine and ²H₂O CoQ and dolichol tracing, MAVEN software (19) was used. For cholesterol, Mass Hunter B.06.00 software was used. Peak area for each isotope was extracted and natural abundance isotope correction performed using an in-house generated algorithm.

For ¹³C mass isotopolog distributions, calculation of fraction newly synthesized cholesterol, dolichol, and CoQ was calculated by dividing each isotopolog by the sum of all isotopologs. Calculation of the fraction newly synthesized cholesterol, dolichol, and CoQ from...
**MEK Inhibition Synergizes with Statins for Cancer Treatment**

**XF Cell Mito Stress analysis**

Oxygen consumption rate (OCR) was determined using an XF96 Extracellular Flux Analyzer (Seahorse Agilent Technologies). Mitochondrial respiratory capacity was determined using XF Cell Mito Stress Kit (Agilent Technologies). Twenty-four hours prior to analysis, cells were seeded in 5% dFBS supplemented base medium containing 10 mM glucose and 2 mM/L glutamine and the indicated drugs or DMSO control. One hour prior to assay, media were replaced with Seahorse media containing 1% dFBS, 10 mM/L glucose, and 2 mM/L glutamine and indicated drugs/DMSO, pH 7.4. During the assay, 1 mM/L oligomycin A, 1 mM/L FCCP, and 0.5 mM/L rotenone/antimycin A were sequentially added.

**NRF2 knockdown**

Cells were plated 12 hours prior to transfection with RNAiMAX (Invitrogen) and siRNAs genomE nontargeting control pool (Dharmacon) and NRF2 (Nfe212; Qiagen, SI01326815, SI01326822), according to the manufacturer’s protocol. After 48 hours, cells were plated and treated as described previously.

**Synergy assay**

Synergy assay

Cells were plated in 24-well plates in base media for 24 hours. Thereafter, media were replaced with base media plus drug or control (DMSO). Simvastatin was used at 0.5, 1, 2, 4, and 8 mM/L. AZD6244 was used at 0.25, 1, 2, 5, 10, 15, and 20 mM/L. A combinatorial matrix of these concentrations was then tested. An Incucyte Zoom (Essen Bioscience) was used to image wells and confluence after 96 hours was determined using Incucyte Zoom Software (Essen Bioscience). Confluence data were normalized to the vehicle (DMSO) condition. For each drug alone, using Microsoft Excel, the confluence curve was fitted to a cubic equation. The cubic equation was then used to create a lookup table of percent inhibition versus drug concentration. The ICX for a given X was then obtained finding the X value in the percent inhibition column and retrieving the associated drug concentration. For a given X and drug concentrations D1 and D2, the drug combination index (CI) was then calculated as described by Chou-Talalay (22), using the following equation:

$$CI = \frac{D_A}{IC_{X,A}} + \frac{D_B}{IC_{X,B}}$$

Where $D_A$ and $D_B$ are the concentration of simvastatin and AZD6244 used in combination to achieve X % drug effect. $IC_{X,A}$ and $IC_{X,B}$ are the concentrations of simvastatin and AZD6244 as single agents to achieve the same effect. A CI of less than 1 indicates synergy.

**Real-time quantitative PCR**

RNA was extracted using the RNAeasy Kit (Qiagen) and cDNA synthesized using Quantitect Reverse Transcription Kit (Qiagen). SYBR Green Master Mix (Bio-Rad) was used to prepare PCR reaction mixtures containing 1 μg of cDNA. A CFX96 thermal cycler (Bio-Rad) was used to perform the PCR reaction. Tubulin was used as a reference gene for mouse and actin for human samples.

**DCFDA assay**

2',7'-Dichlorofluorescin diacetate (DCFDA) was used to measure intracellular ROS using the DCFDA Cellular ROS Detection Assay Kit (ab113851, Abcam) according to the manufacturer’s protocol. Cells were treated for 24 hours in the indicated conditions prior to assay in base media containing 5% dFBS, 10 mM/L glucose, and 2 mM/L glutamine and indicated drugs/DMSO with no phenol red. A Tecan SPARK plate reader with excitation/emission wavelengths filter: 490/510–570 nm was used to detect fluorescence. Average relative fluorescence of control was equated to 100%, with treatment conditions calculated proportionally. Signal was background corrected and adjusted to cell number.

**IHC**

IHC of formalin-fixed paraffin-embedded tumor and benign pancreas blocks was obtained from cohort mice treated with vehicle, simvastatin, or simvastatin + AZD6244. The following primary antibodies were used: phospho-histone H2AX (Ser139; 20E3; γH2AX; Cell Signaling Technology) at 1:50 dilution, anti-8-Hydroxy-2'-deoxyguanosine (N45.1; 8-hydroxy; Abcam) at 1:200 dilution, and caspase-3 (Cell Signaling Technology) at 1:500 dilution. Antigens were retrieved in a PT Module (Agilent) for 25 minutes at 98°C in PT Module 1 buffer (Thermo Fisher Scientific). For phospho-Histone H2AX and caspase-3, endogenous peroxidase activity was blocked by incubation with 3% H2O2. This step was carried out after anti-8-hydroxy-2'-deoxyguanosine staining, so as not to disrupt ROS. Staining was performed on a Dako Autostainer Link48 (for 8-hydroxy and γH2AX) and a Leica Bond RX Autostainer (caspase-3) with the primary antibody applied for 45 minutes at room temperature and 30 minutes, respectively. Sections were washed with Tris-buffered Tween (TBT) and Rabbit EnVision (Agilent) was applied for 30 minutes, before washing with TBT and then Liquid DAB (Agilent) was applied for 10 minutes. Sections were rinsed in water on an autostainer and counterstained with hematoxylin, nuclei blue’d, before being dehydrated and cleared through graded alcohols and xylene before application of a permanent coverslip.

**Results**

**Statins only modestly affect cholesterol pools, but robustly block coenzyme Q synthesis in cancer cells**

Cells acquire cholesterol either through uptake of lipoproteins from the extracellular environment or by synthesizing it de novo (Fig. 1A; ref. 23). In pancreatic and prostate cancer, cholesterol metabolism genes in particular have been shown to be deregulated (8, 24). Cholesterol synthesis itself has not been measured before in these cancer types, and therefore we explored cholesterol dynamics as well as the effect of statins.

We performed a stable isotope tracing experiment with both [U13C]-glucose and [U13C]-glutamine in a cancer cell line derived from the KPC (Pdx1-Cre; KrasG12D/12; Trp53R172H/1) genetically engineered mouse model of pancreatic ductal adenocarcinoma (PDAC). In the presence of 5% (high) serum, approximately 90% of the cholesterol remained unlabeled and very little 13C accumulated in the 27-carbon molecule (Fig. 1B; Supplementary Fig. S1A), thus indicating minimal de novo synthesis, but robust uptake of (unlabeled) cholesterol from the medium. As serum is a source of unlabeled cholesterol, we next lowered extracellular cholesterol by reducing serum to 2% (low; Fig. 1B; Supplementary Fig. S1A). While the fraction of unlabeled (M0) cholesterol remained substantial, the presence of heavily 13C-labeled isotopologs now demonstrated active cholesterol synthesis (Fig. 1B). Of note, we observed partially labeled molecules that contain both 12C and 13C. This is still indicative of complete de novo cholesterol synthesis, and is caused by partial
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labeling of the acetyl-CoA pool, which is also observed for other macromolecules (17). On the basis of this labeling pattern, uptake (M0) and synthesis (M1 –M5) contributed roughly equally to the cholesterol pool (Supplementary Fig. S1A). A similar observation was made in the human PDAC cell line Mia-PaCa2 (Supplementary Fig. S1B–S1D). These results demonstrate that cancer cells preferentially take up cholesterol from their extracellular environment, but can maintain cholesterol homeostasis by inducing de novo synthesis when extracellular availability is limited. This homeostasis is maintained during statin treatment (Supplementary Fig. S1D); simvastatin treatment in low serum conditions potently abrogated de novo cholesterol synthesis, but cells were still able to adapt and maintain their cholesterol pool size by increasing cholesterol uptake (Supplementary Fig. S1D); simvastatin treatment during statin treatment (Supplementary Fig. S1D); simvastatin treatment in low serum conditions potently abrogated de novo cholesterol synthesis, but cells were still able to adapt and maintain their cholesterol pool size by increasing cholesterol uptake (Supplementary Fig. S1D). This finding was recapitulated with pitavastatin and atorvastatin, in KPC, Mia-PaCa2, and a human prostate cancer cell line, PC3 (Supplementary Fig. S1I). As cholesterol synthesis is active in low serum but inactive in high serum, this suggests the antiproliferative effect of statins is not mediated through inhibition of cholesterol metabolism. We therefore started exploring other branches of the mevalonate pathway (Fig. 1A). Using LC-MS-based lipidomics, we were able to directly measure both dolichols and coenzyme Q (CoQ), also known as ubiquinone. Dolichols are composed of multiple concatenated 5-carbon isoprenoid units and function to “anchor” glycosylation structures in membranes (25). CoQ is comprised of a benzoquinine ring derived from tyrosine, attached to a 9 (mouse, CoQ9) or 10 (human, CoQ10) isoprenoid-unit tail (Fig. 1D; ref. 26). It functions as an electron carrier in the electron transport chain (ETC), and hence supports oxidative phosphorylation (2).

Analysis of dolichol labeling from [U13C]-glucose and [U13C]-glutamine revealed relatively little 13C-incorporation, and hence a low rate of de novo synthesis (Supplementary Fig. S1H). This contrasted with CoQ, which showed abundant 13C-incorporation as evidenced by the formation of a multitude of 13C-labeled iso-topologs (Fig. 1E). We confirmed that providing 13C-labeled mevalonolactone, a routinely used cell-permeable mevalonate analogue that is rapidly converted upon entry into the cell, and tyrosine, also led to labeled CoQ (Supplementary Fig. S1J–S1K).

In all cell lines tested, statin treatment significantly reduced both de novo synthesis of CoQ and consequently CoQ pool size (Fig. 1F and G; Supplementary Fig. S1L and S1M). It took 24 hours for statins to have a clear effect on CoQ levels, and this effect could be rescued using mevalonolactone (Supplementary Fig. S1N). Together, these results demonstrate that, in contrast to cholesterol, CoQ is actively produced by cancer cells, and that both synthesis and pool size are reduced upon statin treatment.

Decreased CoQ levels cause impaired oxidative phosphorylation and a compensatory shift toward glycolysis

Succinate dehydrogenase (SDH), or complex II, participates both in the TCA cycle and the ETC. It couples the oxidation of succinate with the reduction of CoQ (27). To establish whether diminished CoQ levels post statin treatment inhibited SDH activity, we determined the levels of its direct substrate succinate (Fig. 2A; Supplementary Fig. S2A). Indeed, statin treatment caused significantly higher succinate levels, suggesting inhibition of SDH activity. We next asked whether reduced SDH activity due to statin-mediated CoQ depletion more broadly impacted central carbon metabolism. We therefore measured OCR after 24 hours of statin treatment, as CoQ depletion is strongest at this time (Supplementary Fig. S1N). Simvastatin, pitavastatin, and atorvastatin all reduced both basal and maximal respiration (Fig. 2B; Supplementary Fig. S2B). Thus, reduced CoQ levels lowered oxidative phosphorylation (OXPHOS). Cells can compensate for loss of energy production from the TCA cycle by increasing glycolysis. In accordance with this, we observed increased glucose uptake and lactate secretion (Fig. 2C; Supplementary Fig. S2C). Of note, OXPHOS was restored upon mevalonolactone supplementation (Supplementary Fig. S2D). Thus, cells switch to glycolysis to compensate for loss of OXPHOS-mediated ATP production due to statin-mediated CoQ depletion.

Statin-induced reduction in CoQ levels causes elevated ROS and activation of antioxidant metabolic pathways

Mitochondrial metabolism is intimately linked with ROS maintenance, and dysfunctional oxidative phosphorylation, as we observed with statin treatment (Fig. 2), can be a principal cause for excessive ROS generation (28). In addition, the reduced form of CoQ, also known as ubiquinol, may have antioxidant functions in cells beyond oxidative phosphorylation (29). We next mined our metabolomics dataset to evaluate whether oxidative stress occurs in statin-treated cells. This revealed a striking shift in the ratio between oxidized and reduced glutathione toward the more oxidized form (Fig. 3A; Supplementary Fig. S3A). Thus, it appears that oxidative stress is indeed a major consequence of statin treatment in cancer cells.

In recent years, multiple metabolic adaptations have been shown to have the ability to regulate redox balance (30). These adaptations include (i) the reductive formation of citrate from glutamine for shuttling NADPH into the mitochondria (31), (ii) increased cellular cystine import for glutathione production (32), and (iii) the synthesis of proline as an electron sink and ROS scavenger (31), (ii) increased cellular cystine import for glutathione production. Consistent with this pathway being active, glutamate secretion increased upon statin treatment (Fig. 3D; Supplementary Fig. S3D and S3E). The transcript levels of SLC7A11/Slc7a11 (xCT) for glutathione production. Consistent with this pathway being active, glutamate secretion increased upon statin treatment (Fig. 3D; Supplementary Fig. S3D and S3E). The transcript levels of xCT transporter also increased (Fig. 3G; Supplementary Fig. S3F).

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Finally, proline synthesis by pyrroline-5-carboxylate reductase (PYCR) from glutamine can also help regulate redox balance by regenerating NAD$^+$, which in turn may promote TCA cycling (34). Accordingly, we found that statin treatment robustly increased proline synthesis from glutamine (Fig. 3H; Supplementary Fig. S3G). PYCR1/Pycr1 transcript levels were also elevated (Fig. 3I; Supplementary Fig. S3H). Proline itself can act as a ROS scavenger, reacting with hydroxyl radicals to form hydroxyproline (33, 35). We indeed observed intracellular hydroxyproline levels to be significantly increased upon statin treatment (Fig. 3J; Supplementary Fig. S3I). Thus, proline synthesis from glutamine helps to regenerate NAD$^+$ and facilitate ROS scavenging.

We next asked whether the effects of statin-induced redox stress could be alleviated by mevalonolactone supplementation (Fig. 3G and I; Supplementary Fig. S3A, S3C, and S3E–I). Indeed, cell proliferation was rescued by mevalonolactone supplementation, as well as by the antioxidant N-acetyl cysteine addition (Supplementary Fig. S3J). Of note, direct CoQ supplementation did not rescue cell growth, but this could have been caused by the precipitation of CoQ in the culture medium, and we were unable to confirm uptake or use by the cells. Nevertheless, the ability of N-acetyl cysteine to rescue the effect of simvastatin is further evidence that the depletion of CoQ and resulting oxidative stress is an important mechanism of statin-induced cell death. Overall, our results indicate that treating cancer cells with statins leads to pronounced oxidative stress and cells compensate by activating redox-induced metabolic pathways that mitigate ROS damage.
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Figure 3.
Statin treatment causes increased redox stress and metabolic compensation. A, Effect of simvastatin (Simva) on the ratio of oxidized (GSSG) to reduced (GSH) glutathione (48 hours). Relative to vehicle control (DMSO). B, Overview of metabolic pathways mitigating oxidative stress. C, Glutamine uptake and glutamate release rates in response to vehicle or simvastatin (2 μmol/l). D, Citrate labeling from [U-13C]-glutamine tracing for 48 hours. E, Change in cystine uptake in response to simvastatin treatment. Rates are relative to vehicle control. F, Effect of simvastatin treatment on intracellular cystine levels (48 hours). V, Change in cystine uptake in response to simvastatin (Simva) + mevalonolactone (Mev.; 48 hours). Values are relative to control. G, qPCR analysis of xCT following statin treatment and statin plus mevalonolactone (Mev.; 48 hours). H, Proline labeling from [U-13C]-glutamine after statin treatment and statin plus mevalonolactone (Mev.; 48 hours). J, Intracellular hydroxyproline levels in response to statin treatment. Peak areas were normalized to cell numbers and expressed relative to control. All data shown as mean ± SEM of three biological replicates (A, D, F, and J), each performed in triplicate, one representative biological replicate, each performed in triplicate (C, E, and H), and three biological replicates from six technical repeats (G and I). Statistical significance was tested by ANOVA for G and I, rest by t test. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. See also Supplementary Fig. S3.
Statin treatment decreases CoQ synthesis and causes oxidative stress in vivo

We next evaluated mevalonate pathway activity in vivo using 2H2O tracing, which enables persistent labeling of macromolecules (56, 57). Pdx1-Cre; KrasG12D+/−, Tp53R172H+/− (KPC) mice were administered a 0.035 mM/L mouse weight 2H2O bolus to mice with palpable tumors, followed by exposure to 8% 2H2O in drinking water for 7 days, which consistently led to approximately 5% deuterium enrichment in plasma (Supplementary Fig. S4A).

We assessed 2H-enrichment in cholesterol. A fractional enrichment of approximately 0.45 was observed in both liver and plasma of KPC mice (Supplementary Fig. S4B; Supplementary Fig. S4C). In healthy pancreas and tumors, the fractional enrichment of cholesterol was comparable with what was observed in plasma (Supplementary Fig. S4D), suggesting that the de novo synthesis of cholesterol in tumors is minimal, as tumors actively take it up from the bloodstream. Statin treatment had no significant impact on cholesterol labeling (Supplementary Fig. S4C) or levels, which is consistent with reports that statins do not affect circulating cholesterol (Supplementary Fig. S4J), indicating that cholesterol was comparable with what was observed in plasma (Supplementary Fig. S4D). Analysis of in vivo dolichol labeling revealed a similar labeling pattern to cholesterol, with labeling in tumors somewhat lower than in plasma, suggestive of minimal synthesis in the tumor, with no observable effect of simvastatin (Supplementary Fig. S4E and S4F).

We next proceeded with analysis of CoQ. Notably, CoQ 2H labeling in the small intestine was very high (fractional enrichment ~ 0.9), presumably due to the extremely rapid turnover of the epithelium (Supplementary Fig. S4G). Fractional enrichment of circulating CoQ was strikingly similar to cholesterol (Supplementary Fig. S4H). In contrast, while CoQ labeling in healthy pancreas was lower than circulating CoQ, the inverse was true in KPC PDAC tumors, where enrichment was significantly higher (Fig. 4A). As the enrichment is higher than circulating CoQ, this indicates active synthesis in the tumor. This was significantly reduced upon statin treatment (Fig. 4A). Notably, plasma CoQ9 levels were not significantly altered by statin treatment, indicating a tumor-specific effect (Supplementary Fig. S4I), which appears in contrast to studies in healthy humans (40). Nevertheless, we conclude that PDAC tumors actively synthesize CoQ, and this synthesis is reduced by statin treatment.

We next wanted to investigate whether simvastatin treated tumors also had elevated ROS levels. Indeed, our IHC demonstrated elevated oxidative damage, as there was significantly greater staining of 2H2AX in the simvastatin-treated cohort (Fig. 4B). We used 8-hydroxyguanosine as a DNA damage marker (41), and found significantly more stained cells in the simvastatin-treated cohort than vehicle treated (Supplementary Fig. S4J). Consistent with the metabolic compensation observed in vitro, we found that metabolic compensation to mitigate oxidative stress occurred in the PDAC tumors. Specifically, we observed a significant increase in xCT (Slc7a11) transcript levels (Fig. 4C). Thus, simvastatin treatment leads to increased tumor ROS and tumors compensate by attempting to elevate glutathione production via xCT upregulation.

Combined statin and MEK inhibitor treatment synergize to accumulate ROS and cause apoptosis

Our findings revealed that statins reduce CoQ levels, causing oxidative stress, and metabolic compensation occurs to mitigate the effects of ROS damage. We next asked whether disrupting these metabolic adaptations, together with statin treatment, would synergize to induce tumor cell death. The most pronounced metabolic adaptation we observed in vivo was upregulation of the xCT transporter (Fig. 4C). It was previously shown that the MEK inhibitor AZD6244 can promote ROS by reducing NRF2 induction (42). In line with this, we found simvastatin treatment increased NRF2 transcript levels, while AZD6244 treatment reduced NRF2 transcript levels, as expected (Supplementary Fig. S5A). As xCT is a NRF2 target (43) and NRF2 itself is regulated by MEK (44), we sought to determine whether the ROS-promoting effect of AZD6244 was through lowered expression of xCT. Indeed, AZD6244 was able to reduce xCT transporter levels in cultured cancer cells (Fig. 5A). We further validated this by testing MEK targets and found them to be reduced (Supplementary Fig. S5B).

Specifically, our previous results showed simvastatin elevates xCT mRNA levels, but in combination with AZD6244 this was prevented and the expression even reduced below the vehicle condition (Fig. 5A). This resulted in reduced cystine uptake (Supplementary Fig. S5C), indicating that cells were not able to compensate for the elevated ROS from simvastatin treatment through increased cystine uptake for glutathione synthesis. In accordance, ROS levels were significantly increased using the combination treatment (Fig. 5B). There was also a trend toward increased glutathione oxidation after combination treatment (Supplementary Fig. S5D). Overall the combination treatment exacerbates ROS levels. Importantly, proliferation was substantially reduced upon combination treatment and NRF2 knockdown (KD; Fig. 5C; Supplementary Fig. S5E and S5F). NRF2 KD reduced NRF2 target transcript levels, and most notably xCT levels (Supplementary Fig. S5G). Combined, these results suggest AZD6244 may be synergizing with simvastatin.

To explore the potential synergy of AZD6244 and simvastatin further, the effect upon cell proliferation of the two drugs as single agents and in combination was analyzed (see Supplementary Fig. S5H and S5I for example graphs). Using a matrix of all drug concentration combinations, the combination index (CI), as described by Chou (45), we sought to determine whether the ROS-promoting effect of AZD6244 was through lowered expression of xCT. Indeed, AZD6244 was able to reduce xCT transporter levels in cultured cancer cells (Fig. 5D). This shows AZD6244 is able to counter the elevated xCT levels induced by statin treatment in the tumor setting (Fig. 5E). Strikingly, our IHC revealed caspase-3 induction and associated apoptotic body numbers were significantly higher in the combination treatment compared with vehicle, AZD6244, or simvastatin alone (Fig. 5F and G). Combined, these results indicate that a combined treatment with statin and a MEK inhibitor may be an effective cancer treatment paradigm.

Discussion

Our rationale for studying the mevalonate and downstream pathways in cancer was 2-fold. First, while the mevalonate pathway is known to be upregulated in a number of different cancer types (6, 8, 10), and to be critical for tumorigenesis (9), what downstream products are known to be upregulated in a number of different cancer types (6, 8, 10), and to be critical for tumorigenesis (9), what downstream products are synthesized and how they contribute to tumorigenesis, thus far remained largely unexplored. Second, despite the importance of the
mevalonate pathway in cancer, the chemotherapeutic potential of the ubiquitously prescribed statins, either as a single agent or in a combination strategy, remained uncertain. We combined both GC-MS and LC-MS modalities with innovative stable isotope tracing approaches to determine the metabolic activity of this branch of metabolism, as well as the compensatory mechanisms that occur upon pharmacologic inhibition.

It is well established that statins lower circulating cholesterol levels in humans, yet few studies have looked at cholesterol metabolism directly in cancers (8, 45). Using tracers, we directly measured cholesterol metabolism, both in vitro and in vivo, to reveal that cancer cells preferentially take up cholesterol, rather than synthesizing it. Cholesterol uptake occurs via the LDL receptor and its increased expression has been reported in a variety of tumor types, including pancreatic cancer (8).

Although our labeling studies clearly demonstrated that cholesterol synthesis is minimal in tumor cells, statin treatment still elicited a robust antiproliferative response. Through further exploration using an innovative combination of stable isotope tracing and lipidomics, we discovered that CoQ is actively synthesized by cancer cells. This contrasted with the synthesis of dolichols, which showed substantially less pronounced labeling. The principal function of
MEK Inhibition Synergizes with Statins for Cancer Treatment

Figure 5.
A MEK inhibitor (AZD6244) synergizes with statin treatment to induce cell death due to excessive ROS. A, qPCR analysis of xCT expression following 48-hour treatment with simvastatin, AZD6244, or combination. B, Intracellular ROS as measured by DCFDA assay after 24-hour treatment with simvastatin, AZD6244, or a combination. C, Cell numbers following 96-hour exposure to indicated conditions. Numbers are relative to vehicle (DMSO)-treated control. D, qPCR analysis of xCT (Slc7a11) expression in KPC PDAC tumors. E, Schematic to show AZD6244 action reducing xCT level. F, Caspase-3 IHC and scoring of KPC mice treated with vehicle control, simvastatin, AZD6244, or AZD6244 + simvastatin. G, Hematoxylin and eosin IHC and scoring of apoptotic bodies in KPC mice treated as in F. Arrows, apoptotic bodies. Scale bar in larger image, 50 μm; smaller image, 20 μm. Data shown as mean ± SEM of three biological replicates, with each 6 technical replicates (A), three biological replicates, with each 8 technical replicates (B), three biological replicates, each performed in triplicate (C). Data are shown for n = 4 mice per group (D, F, and G). In F and G, quantification is shown as median score ± SEM from 20 frames. Statistical significance was tested by ANOVA for A–D or Mann–Whitney for F–G, *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. See also Supplementary Fig. S5.
CoQ is to act as an electron carrier in the electron transport chain (ETC), to facilitate mitochondrial respiration. Apart from distinct oncogenic mutations and deletions in TCA cycle enzymes (46), mitochondrial metabolism is typically active in tumor cells and an important source for both energy and building blocks for macromolecules (47). In fact, multiple recent reports demonstrated heightened glucose oxidation in tumors (48, 49), stressing the importance of mitochondrial metabolism in cancer.

Statin-mediated CoQ depletion causes severe oxidative stress, which is likely caused by the disruption in mitochondrial metabolism. A few reports have previously postulated a link between statins, reduced CoQ levels, and increased ROS in other cell types, and this has been suggested as a cause for statin-induced myopathy (50, 51). However, the published data supporting this was circumstantial. We now establish this link in unprecedented detail and show it also occurs in cancer cells. Furthermore, we made the novel observation that statin-mediated CoQ depletion leads to the compensatory induction of multiple metabolic pathways, with each having a unique antioxidant role. Particularly pronounced in both in vitro and in vivo settings was the upregulated expression of the xCT transporter. This has previously been shown to occur in response to oxidative stress by helping cells to obtain cystine needed for glutathione production (52). Of note, the statin-mediated effect on ROS through CoQ is distinctly different from recent reports on squalene, which accumulates in some cancers and has an antioxidant function (53, 54).

Our study highlights the capability of statins to inhibit CoQ synthesis. However, we recognize that this may not be the sole chemotherapeutic effect of statins. Multiple elegant reports have highlighted the pronounced effect of statins on protein prenylation, a posttranslational modification that occurs on prominent oncoproteins, including members of the RAS family (55, 56). Recent evidence, however, clearly demonstrated that the anticancer effects of statins is not due to reduced RAS protein prenylation (12). Therefore, other statin-induced alterations, including the pronounced ROS production due to CoQ loss, contribute to its chemotherapeutic potential.

Statins, extensively prescribed for cardiovascular disease, have been widely evaluated for their effects on tumor development and progression, yet their clinical effect is variable (57–59). A phase II clinical trial of simvastatin and gemcitabine in patients with PDAC found no clinical benefit to combining the statin with the only chemotherapy currently available for patients with pancreatic cancer (60). Similarly, for prostate cancer, a recent randomized double blind trial found no significant difference between atorvastatin and placebo (61). We explored statins in both pancreatic and prostate cancer cell lines, as well as an in vivo genetically engineered mouse model of PDAC. Both in vitro and in vivo simvastatin reduced CoQ synthesis significantly and tumors exhibited elevated ROS levels and xCT transcript levels. This led us to target the compensatory xCT upregulation using the MEK inhibitor AZD6624 and we found this dual combination with simvastatin-induced apoptosis in the tumor.

Our findings are directly relevant to unearthing a potential combinatorial therapy for these cancers by targeting metabolic compensation in response to excessive ROS generation following statin treatment.

We urgently need better treatments to target aggressive cancers such as PDAC, for which therapeutic options are currently very limited. We have used two FDA-approved drugs and demonstrated their synergy and potential as a combinatorial cancer therapy.

Disclosure of Potential Conflicts of Interest

J.J. Kamphorst is Director of Cellular Metabolism for Rheos Medicines and has ownership interest (including stock, patents, etc.) in the same. No potential conflicts of interest were disclosed by the other authors.

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References


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Targeting the Metabolic Response to Statin-Mediated Oxidative Stress Produces a Synergistic Antitumor Response

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