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**Title:** Mechanism of action of statins in colorectal cancer  
**Issue Date:** 2013-10-10
CHAPTER FIVE

KINOME-WIDE ANALYSIS OF THE EFFECT OF STATINS IN COLORECTAL CANCER


Submitted
TRANSLATIONAL RELEVANCE

Statins are one of the most prescribed class of drugs worldwide and given their relative safety and their beneficial effects on overall survival, they are attractive candidates for use as chemopreventive agents in colorectal cancer (CRC). Despite considerable epidemiological evidence that statins have beneficial effects in CRC their molecular mechanism of action in CRC remains unclear. In this article we explore the effect of statins on the kinome in CRC cells in a hypothesis free manner. We show that statin treatment of CRC cells leads to widespread changes in kinase activity affecting multiple signaling pathways involved in oncogenesis. This broad, hypothesis-free approach and the confirmation of the major effects in mice and pilot experiments in humans, provides new insights into the working mechanisms of statins, which may facilitate their rational, targeted use in CRC.

ABSTRACT

Purpose: Epidemiological studies and meta-analyses show an association between statin use and a reduced incidence of colorectal cancer (CRC). We have shown that statins act on CRC through Bone Morphogenetic Protein (BMP) signaling, but the exact cellular targets and underlying mechanism of statin action remain elusive. In this study, we set out to assess the influence of statins on global cancer cell signaling by performing an array-based kinase assay using immobilized kinase substrates spanning the entire human kinome.

Experimental Design: CRC cells with or without statin treatment were used for kinome analysis. Findings on kinome-arrays were further confirmed by immunoblotting with activity-specific antibodies. Experiments in different CRC cell lines using immunoblotting, siRNA-mediated knockdown and treatment with specific BMP-inhibitor Noggin were performed. The relevance of in vitro findings was confirmed in xenografts and in CRC patients treated with statins.

Results: Kinome analysis can distinguish between non-specific, toxic effects caused by 10 µM of Lovastatin and specific effects on cell signaling caused by 2 µM Lovastatin. Statins induce upregulation of PTEN activity leading to downregulation of the PI3K/Akt/mTOR signaling. Treatment of cells with the specific BMP inhibitor Noggin as well as PTEN knockdown and transfection of cells with a constitutively active form of AKT abolishes the effect of Lovastatin on mTOR phosphorylation. Experiments in xenografts and in patients treated with statins confirm statin-mediated BMP pathway activation, activation of PTEN and downregulation of mTOR signaling.

Conclusions: Statins induce BMP-specific activation of PTEN and inhibition of PI3K/Akt/ mTOR signaling in CRC.
CHAPTER FIVE

INTRODUCTION

Colorectal cancer (CRC) is a major public health problem worldwide and incidence rates are increasing, especially in areas historically at low risk (1). Despite improvements in treatment, advanced disease remains incurable and hopes to achieve reductions in mortality are pinned on nationwide screening programs aimed at early detection and prevention. Removal of cancer precursor lesions at colonoscopy is the foundation of CRC prevention but offers far from perfect protection from CRC (2–4). CRC prevention using chemical or natural compounds (chemoprevention) represents a promising alternative or adjuvant preventative strategy (5, 6). However, toxicity issues and the question of risk versus benefit have hampered widespread clinical implementation of chemopreventative drugs (7, 8). Epidemiological studies and meta-analyses have shown an association between the use of 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitors (statins) and a reduced incidence of CRC (9–11). Statins are an attractive candidate for use as a chemopreventive agent in CRC as they are generally well tolerated and have proven beneficial effects on cardiovascular outcomes, improving any overall benefit/risk ratio (12–14). Their exact cellular targets and underlying mechanism of action in CRC are however largely unknown. Moreover, many in vitro studies are performed with high, non-physiological concentrations of statins, and it is not known whether the changes shown are specific and represent true mechanisms of statin action, or are a non-specific result of toxic concentrations of statins on cellular metabolism (15).

We have previously shown that statins act on CRC through activation of Bone Morphogenetic Protein (BMP) signaling (16); (17), but the mechanistic details as to how statin-dependent activation of BMP signaling influences CRC cells remains unknown and there are several other proposed molecular mechanisms of action (18, 19). Kinome profiling using array-based kinase assays has been previously used to comprehensively assess changes in signal transduction in various cell systems including cancer (20, 21).

In this study, we assess the influence of statins on global cell signaling in a hypothesis-free manner. To create a full picture of the activity of different signaling pathways after statin treatment we performed an array-based kinase-assay using immobilized kinase substrates spanning the entire human kinome (22, 23). From patterns of substrate phosphorylation we used established algorithms to deduce global cellular kinase activity in human CRC cells with or without Lovastatin treatment. We have shown toxic changes in signal transduction due to high 10µM concentration of statins and established that a concentration of 2µM, a concentration seen in the serum of patients treated with statins (24), leads to specific, logical sequential changes in cellular signaling. We show that statin treatment leads to widespread changes in kinase activity in cancer cells compatible with anti-tumor activity with most pronounced effects on PI3K/AKT/mTOR signaling. We further confirm these data by a series of in vitro experiments and show that the inhibiting effect of statins on the
AKT/mTOR pathway occurs via PTEN in a BMP-dependent manner. Finally, experiments in xenografts and in patients with CRC treated with statins confirm that the changes in the BMP pathway and downregulation of phospho- mTOR seen cell lines in vitro also occur in tumors in vivo.

MATERIALS AND METHODS

Cell Culture
HCT116, RKO and HT29 colon cancer cell lines were obtained from the American Type Culture Collection and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Life-technologies, Breda, the Netherlands) containing 4.5 g/L glucose and L-glutamine, penicillin (50 U/mL) and streptomycin (50 μg/mL) (Life-technologies), and 10% fetal calf serum (Life technologies). Cells were grown in monolayers at 37 °C under a humidified 5% CO2 atmosphere. Lovastatin (M2147, Sigma Aldrich, Zwijndrecht, Netherlands) was dissolved in 100% Ethanol (Sigma-Aldrich) and Noggin-Fc (719-NG, R&D Systems Europe Ltd, Abingdon, United Kingdom) was dissolved in sterile PBS and both were stored in 500μl aliquots at -20°C until required.

Phosphoproteome determination
Cells were trypsinized on ice, spun down and washed in Phosphate-buffered saline (PBS). Cells were lysed in a non-denaturing complete lysis buffer (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L Na2EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L MgCl2, 1 mmol/L beta-glycerophosphate, 1 mmol/L Na3VO4, 1 mmol/L NaF, 1 μg/mL leupeptin, 1 μg/mL aprotinin, 1 mmol/L phenylmethysulfonyl fluoride) and the volume of the cell lysate was equalized with distilled H2O. Activation mix (10 μL), containing 50% glycerol, 50 μM ATP, 60 mm MgCl2, 0.05% (vol/vol) Brij-35, 0.25 mg/ml BSA, and 2000 μCi/ml γ-33P-ATP was added to 60 μL cell lysate. The peptide arrays (Pepscan, Lelystad, the Netherlands), containing triplicates of 1024 different kinase substrates (992 substrates, 32 controls), were incubated with the cell lysates for 2 hours in a humidified stove at 37°C plus 2000 μCi/ml 33P-γ-ATP or 33P-α-ATP (control for non-covalent specific binding). Subsequently, the arrays were washed twice with Tris-buffered saline (TBS) with 0.1% Tween-20, twice in 2mol/L NaCl, twice in demineralized H2O, and then air-dried. The arrays were exposed to a phospho-imaging screen for 72 hours and scanned on a phospho-imager (Storm™, Amersham-Biosciences, Uppsala, Sweden).
Analysis of Peptide Array

Spot density and background were analyzed using Scanalyze (http://rana.lbl.gov/EisenSoftware). Spot intensities were normalized to the 90th percentile (The 5% and 95% intervals of the spot density were calculated for each data set, and all spots were normalized within these boundaries so that the 5% and 95% levels are given the values 0 and the 100 respectively). The Spearman correlation coefficient was calculated for each combination of sets and clustering was performed using Johnson hierarchical clustering schemes (26).

For each peptide the average and standard deviation of phosphorylation was determined and plotted in an amplitude-based hierarchical fashion. Background phosphorylation of the array was determined from the exponent describing the amplitude behavior of the 500 least phosphorylated peptides (which are assumed not to contain phosphorylation derived from a relevant biological signal). Peptides of which the average phosphorylation minus 1.96 times the standard deviation was higher than background were considered to represent true phosphorylation events (p < 0.05). Peptide sets which showed statistically significant changes compared to the background were subsequently subjected to elective Markov analysis. (Tables S1A and S1B) Kinase activities in lysates from cells incubated with or without Lovastatin were determined by significant fold change ratios of the combined values of phosphorylated peptides resembling a substrate for kinase activity. Significance analysis was performed using a minimal modification for the algorithm originally developed for microarray analysis (www-stat.stanford.edu/~tibs/SAM/)(25).

Immunoblotting

Cells at 60%-80% confluence from 6-well plates (Greiner, Alphen a/d Rijn, the Netherlands) were washed in ice-cold PBS and scraped into 200µL of cell lysis buffer (Cell Signaling, Leiden, the Netherlands) containing a protease inhibitor cocktail (MP-Biochemicals, Illkirch, France). Protein concentration was measured using the RC DC protein assay kit (Bio-Rad, CA, USA) according to the manufacturer’s instructions and the protein concentrations of the samples were equalized accordingly. Samples were diluted in 3x sample buffer (125 mmol/L Tris/HCl, pH 6.8; 4% sodium dodecyl sulphate (SDS); 2% β-mercaptoethanol; 20% glycerol, 1 mg bromophenol blue), sonicated and then heated at 95° for 5 minutes. 50 µg of protein from each sample was loaded onto SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) membrane (Millipore, MA, USA). The blots were either immersed in methanol for 10 seconds and blocked by air drying for 1 hour at room temperature, or incubated in blocking buffer (TBST [Tris-buffered Saline with 0.05 % Tween] with 5% low-fat milk powder) for 1 hour. The membranes were subsequently washed 3 × 10 minutes in TBST before overnight incubation at 4°C with primary antibody in primary antibody buffer (TBST with 0.2% low-fat milk powder). Blots were washed 3 × 10 minutes in TBST and incubated for 1 hour at room temperature in the appropriate secondary antibody (1:1000) (Dako, Glostrup, Denmark) in blocking buffer. After 3× 10 minute washing in TBST, blots
were incubated with Lumi-light (Roche, Woerden, the Netherlands), and chemiluminescence was detected using a VersaDoc imaging system (Bio-Rad).

Antibodies
p-Akt (473 and 308), p-mTOR, mTOR, p-PTEN, PTEN, p70-S6K, p-4EBP1, p-Ask1, p-MAPK-p38, p-MAPK-JNK, p-LKB1, AMPK, pMAPK-Erk (p44-42), and p-SMAD1,5,8 were purchased from Cell Signaling Technology, Inc. (Boston, MA, USA), BMPR1A and β-Actin from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A).

RNA interference
Cells were plated in 12 well plates and treated with siRNA for PTEN or scrambled control siRNA (Cell Signaling, Boston, MA, USA) using DharmaFECT 2 (Dharmacon, Epsom, UK) as described by manufacturer for 48 hours. Immunoblotting was performed for efficiency control.

RNA Isolation and cDNA Synthesis
For isolation of RNA from cells we used the RNeasy Kit (Qiagen, Netherlands) according to the manufacturer’s protocol. Complementary DNA was made using the RevertAid M-MuLV Reverse Transcriptase (Fermentas, St-Leon-Rot, Germany) according to the manufacturer’s protocol.

Immunohistochemistry
Formalin-fixed, paraffin-embedded tissue blocks were sectioned (4 μm), deparaffinized, immersed in 0.3% H2O2 in methanol for 30 min and heat treated at 100°C (pH 6.0) for 20 min. Sections were blocked with TENG-T (10 mmol/l Tris, 5 mmol/l ethylenediaminetetraacetic acid, 0.15 mol/l NaCl, 0.25% gelatin, 0.05% (vol/vol) Tween 20, pH 8.0) for 30 min. Slides were incubated with primary antibodies to pSMAD1,5,8 (1:50), pPTEN (1:50) and to pmTOR (1:50) overnight at 4°C. The LSAB+ System-HRP (Dako) was used to visualize the antibody-binding sites. Peroxidase activity was detected with 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). Sections were counterstained with hematoxylin (Sigma-Aldrich). All stainings were evaluated by experienced pathologists (LK/HM). Finally, the stains were analyzed using ImageJ software.(27)

Xenograft mouse model
Two groups of 8 female NMRI nu/nu mice were injected subcutaneously in the flank with 1 × 106 HCT116 cells or with 5 × 106 HT29 cells in Matrigel (BD Bioscience, Breda, Netherlands). Mice were fed ad libitum with food containing Simvastatin (Arie-Blok-BV, Woerden, Netherlands), thereby receiving 50 mg/kg/day for 3 weeks, initiated when the tumor volume reached 100–200 mm3. After the mice had been sacrificed, the tumors were
harvested and embedded into paraffin blocks. Simvastatin was chosen as it is the second most potent statin in our in vitro experiments (16), and is licensed for use in humans in the Netherlands whereas Lovastatin is not.

Selection of patient material
Four patients newly diagnosed with CRC by endoscopy were treated with 40 mg Simvastatin for 21 days after which elective surgery was performed. Biopsies of the tumor taken during initial endoscopy and tumor tissue collected immediately after surgical removal were formalin-fixed and embedded in paraffin for further analyses. All patients had no previous history of CRC, had never used any statin or used any NSAID during diagnosis or within the previous 6 months. The study was approved by the LUMC Medical Ethics Committee.

Statistical analysis
Statistical analysis was performed using 2-tailed Student’s t test, and p < .05 was considered statistically significant. Asterisks indicate a significant difference between two groups (*p < 0.05; **p < 0.005). All experiments were done with a minimum of 3 independent experiments. Data are shown as mean ± SEM.

RESULTS

The clinically relevant concentration of 2µM of Lovastatin is optimal for analysis of statin-induced signal transduction.

It is not well known whether the high concentrations of medicines which are often used in vitro studies are relevant and reflect the real changes which occur in vivo. Previous in vitro studies have used doses of statins between 0.1 and 667 µM (28-30), while concentrations between 2 µM have been measured in serum of patients treated with statins(24). Statins seem to have rapid dose-dependent effects on cellular signaling and apoptosis observed within 24 hours of treatment, while effects on gene expression were mainly observed after 72 hours of treatment (17). However, at higher doses and with longer treatment statins also lead to extensive cell death which could be not only due specific statin-mediated but also due to more non-specific toxic effects. First, we decided to establish the concentration and duration of statin treatment optimal for investigation of the global cell signaling when only specific, not toxic changes can be demonstrated. HCT116 cells were treated with different Lovastatin concentrations (0.2, 2 and 10µM) and cell viability was assessed at various time points. MTT analysis shows no changes in cell viability after 24 hours of treatment with 0.2 and 2 µM of Lovastatin, but significant reduction in cell viability is observed after 24 hours when a concentration of 10 µM is used. After 48 hours of exposure reduced viability is observed in cells treated with 2 µM and 10 µM of Lovastatin. After 72 hours of treatment 0.2, 2
and 10 µM of Lovastatin induce significant reduction in viability. (Figure 1). We chose to use 24 hour exposure to 2 and 10µM for further analysis to observe and compare the changes in kinome profiles between low and high concentration treatment. PepChip analysis after exposure to 10µM for 24 hours leads to chaotic changes in substrate phosphorylation. We see that the phosphorylation of several substrate targets of the same kinase move in different directions. Further, disordered changes are seen by pathway analysis; where increased kinase activity would be expected to lead to downstream kinase activation within the same pathway, our samples treated with 10 µM of Lovastatin show no consistent pattern (data not shown, available by request). In contrast, analysis of samples treated with 2µM of Lovastatin for 24 hours show consistent and logical changes described below (Figure 2, 3 and Suppl table1). Kinome analysis may therefore represent a novel tool for differentiating between non-specific, toxic effects of an intervention and specific effects on cell signaling. Based on these observations we used 2 µM of Lovastatin for 24 hours for kinome profiling. In this timeframe, molecular processes responsible for statin-induced gene expression changes should be fully operational while minimizing secondary knock-on effects due to the gene expression changes themselves.

Figure 1.
Treatment with the clinically relevant dosage of 2 µM Lovastatin for 24 hours is optimal for kinome profiling. HCT116 colon cancer cells were treated with different concentrations of Lovastatin (0, 0.2, 2 and 10 µM) and cell viability was assessed at different time points using MTT assays. Exposure with 0.2 and 2 µM Lovastatin for 24 hours did not lead to a significant reduction in cell viability, but after 48 hours 2 µM did show decreased cell viability. Exposure to 10 µM Lovastatin reduced cell viability after 24 hours.
Statin treatment inhibits the PI3K/Akt/mTOR pathway

To analyze the actual changes in the phosphoproteome associated with Lovastatin treatment, we first determined the number of significantly phosphorylated substrates for each condition. This is done by calculating the average and standard deviation of the substrate phosphorylation for each peptide in each condition (vehicle control versus 2 μM) (full list of results in supplementary tables 1A and 1B) and plotting the values obtained in an amplitude-based hierarchical fashion (Figure 2a). Whereas lysates of HCT116 cells not treated with Lovastatin phosphorylate 222 peptide substrates, the lysates of the cells treated with 2μM Lovastatin phosphorylate 151 substrates (Table S1B). Venn diagrams were constructed to summarize the changes in the phosphoproteome induced by Lovastatin (Figure 2b). Treated and untreated HCT116 cells phosphorylate only 40 common substrates, phosphorylation of the other substrates being unique to either vehicle treated cells or Lovastatin treated cells. Using established algorithms kinase activity was deduced from the pattern of substrate phosphorylation and used to construct provisional signal transduction schemes to detail the effects of Lovastatin on colon cancer cell signal transduction (Figure 3a). As we and others have shown(18), statin treatment affects multiple pathways compatible with an anti-oncogenic effect. The strongest effect of statin treatment is seen on the PI3K/Akt pathway and its downstream target mTOR (Table 1). Immunoblotting using phosphorylation-specific antibodies was used to validate the major changes seen with the kinome analysis (Figure 3b) and confirmed the downregulation of the phosphorylation level of AKT, mTOR and its downstream target 70S6K (Figure 3b).

Table 1 | Evidence for mTOR inactivation in HCT-116 colon cancer cells

<table>
<thead>
<tr>
<th>Peptide used for kinase reaction</th>
<th>Phosphorylation site in protein</th>
<th>Upstream kinase</th>
<th>Phosphorylation 0 μM Lovastatin</th>
<th>Phosphorylation 2 μM Lovastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEPSIYEVSRV</td>
<td>DAPPI1_Thr139</td>
<td>PI3K</td>
<td>11.9×10^{-3} ± 1.2×10^{-3}</td>
<td>&lt;0.10×10^{-3}</td>
</tr>
<tr>
<td>EDLSAYASISF</td>
<td>IRS1_Thr1229</td>
<td>PI3K</td>
<td>17.6×10^{-3} ± 2.3×10^{-3}</td>
<td>1.4×10^{-3} ± 2.2×10^{-3}</td>
</tr>
<tr>
<td>QSKRSTMVGP</td>
<td>PAK3_Thr423</td>
<td>PDK</td>
<td>6.0×10^{-3} ± 0.6×10^{-3}</td>
<td>4.1×10^{-3} ± 3.0×10^{-3}</td>
</tr>
<tr>
<td>RKRPAADDSST</td>
<td>CDK inhibitor Thr157</td>
<td>AKT1</td>
<td>8.9×10^{-3} ± 1.8×10^{-3}</td>
<td>2.2×10^{-3} ± 2.0×10^{-3}</td>
</tr>
<tr>
<td>RGRGSSVGGS</td>
<td>ASK1_Ser83</td>
<td>AKT1</td>
<td>16.2×10^{-3} ± 0.8×10^{-3}</td>
<td>1.2×10^{-3} ± 0.5×10^{-3}</td>
</tr>
<tr>
<td>RARSTLSNERP</td>
<td>Tuberin_Ser93</td>
<td>AKT1</td>
<td>10.0×10^{-3} ± 2.8×10^{-3}</td>
<td>&lt;0.10×10^{-3}</td>
</tr>
<tr>
<td>RSRDPSLMDF</td>
<td>Fanconi anemia Ser1149</td>
<td>AKT1</td>
<td>13.1×10^{-3} ± 1.5×10^{-3}</td>
<td>2.6×10^{-3} ± 2.1×10^{-3}</td>
</tr>
<tr>
<td>RKRPTSGLHP</td>
<td>BRCA1_Ser509</td>
<td>AKT</td>
<td>14.2×10^{-3} ± 0.6×10^{-3}</td>
<td>1.0×10^{-3} ± 2.6×10^{-3}</td>
</tr>
<tr>
<td>RDRSSAPNVH</td>
<td>B-RAF_Ser564</td>
<td>AKT</td>
<td>34.9×10^{-3} ± 5.9×10^{-3}</td>
<td>9.2×10^{-3} ± 13.5×10^{-3}</td>
</tr>
<tr>
<td>DHRYSDTDDTS</td>
<td>PTEN_Ser380</td>
<td>AKT</td>
<td>2.5×10^{-3} ± 2.0×10^{-3}</td>
<td>&lt;0.10×10^{-3}</td>
</tr>
<tr>
<td>MEARNSPVKT</td>
<td>4EBP1_Ser55</td>
<td>mTOR</td>
<td>22.4×10^{-3} ± 4.9×10^{-3}</td>
<td>15.5×10^{-3} ± 13.5×10^{-3}</td>
</tr>
<tr>
<td>SSTDRPYEVK</td>
<td>Mucin1_Ser1227</td>
<td>GSK3β</td>
<td>4.8×10^{-3} ± 6.8×10^{-3}</td>
<td>8.9×10^{-3} ± 2.0×10^{-3}</td>
</tr>
<tr>
<td>NPCTETFTGTL</td>
<td>ASK1_Ser83</td>
<td>ASK1</td>
<td>0.03×10^{-3} ± 2.3×10^{-3}</td>
<td>5.8×10^{-3} ± 1.7×10^{-3}</td>
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Figure 2
A. Venn diagram to summarize the changes in phosphorylated substrates in HCT116 cells with and without Lovastatin treatment. Treatment with Lovastatin leads to an overall reduction in phosphorylation, but also to a change in which substrates are phosphorylated. In HCT116 cells without Lovastatin treatment 222 substrates are significantly phosphorylated. Lovastatin treated cells have 169 significantly phosphorylated substrates. Only 40 substrates are phosphorylated in both conditions.
B. Illustration of PepChip array analysis methodology. PepChip slides contain substrate motifs, which can be phosphorylated by kinases present in biological lysates. The intensity distribution of the resulting phosphorylation contains an aspecific component and a specific component. As illustrated by the depiction of a non-lysate treated slide, the aspecific component can be described by first sorting substrates according to their intensity after which the function $y = a \cdot \ln(x) + b$ (in which $x$ is the substrate number after amplitude-based sorting and $y$ the resulting spot intensity). The values of $a$ and $b$ can be determined from a slide (including those treated with a biological lysate) by determining the exponent best describing the amplitude behavior of the 500 least phosphorylated peptides (which were assumed not to contain phosphorylation derived from a relevant biological signal). Peptides of which the average phosphorylation minus 1.96 times the standard deviation is higher than background are considered to represent true phosphorylation events ($p < 0.05$), given an "ON" call and further analyzed.
Statin induced inhibition of the Akt/mTOR pathway is PTEN dependent

Statin treatment strongly downregulates the activity of the PI3K/Akt/mTOR signaling pathway, but in which manner this inhibition occurs could not be easily deduced from the kinome analysis. PTEN, a phosphatase of phosphatidylinositol-(3,4,5)-trisphosphate (PIP3), which functions as a negative regulator of the Akt/PKB signaling pathway, was also one of the proteins altered in phosphorylation upon statin treatment in kinome arrays (Table 1). Phosphorylation of PTEN induces a conformational change inhibiting attachment of the protein to the membrane thereby negatively regulating its function as a phosphatase (31-33). Immunoblot analysis of HCT116 cells shows a clear reduction of the amount of phosphorylated PTEN after treatment with Lovastatin, confirming the kinome data (Figure 3B). To investigate whether the effect of statins on the mTOR pathway is mediated through PTEN and not by inhibition of mTOR itself, we transiently transfected HCT116 cells with siRNA targeted against PTEN, or scrambled control siRNA and subsequently treated the cells with Lovastatin or vehicle control. Transfection with siRNA against PTEN leads to a strong increase in the amount of phosphorylated mTOR, and also diminishes the inhibitory effect of statin treatment on mTOR phosphorylation (Figure 4a). The activity of AKT, which is directly upstream of mTOR, is controlled by its phosphorylation via PIP3. PTEN converts PIP3 back to PIP2 (phosphatidylinositol 4,5-diphosphate), thereby indirectly reducing AKT activity. To confirm that the inhibition of mTOR signaling is mediated through increasing PTEN activity and mediated via AKT, we transiently transfected HCT116 cells with a constitutively active form of AKT1 (ca-AKT)(34) and subsequently tested the effect of Lovastatin treatment. After transfection with ca-AKT statin treatment no longer results in reduced phosphorylation of mTOR (Figure 4b), showing that statin treatment works upstream of AKT, most likely via PTEN.

Statin induced inhibition of the Akt/mTOR pathway is BMP dependent

The importance of BMP signaling in colorectal cancer has only relatively recently become apparent through the finding of BMPR1a mutations in Juvenile Polyposis and from GWAS studies (35, 36). A link between BMPR1a and PTEN was discovered in Cowden Syndrome, an inherited hamartomatous polyposis syndrome, where mutations in either of these genes can lead to the same phenotype (37). Studies performed in breast cancer cells revealed that treatment with BMP caused increased levels of PTEN activity (38). Whether this applies to CRC is unknown. We have previously shown that statin treatment leads to an increase in BMP signaling (16, 17) and as shown above, that statin increases PTEN activity leading to downregulation of mTOR signaling. To investigate whether the effect of statins on PTEN is dependent on BMP signaling, we pretreated colon cancer cells with the specific BMP inhibitor Noggin (39) before treatment with Lovastatin and analyzed the effect on the phosphorylation of PTEN and mTOR. As measured by immunoblotting, Noggin not only abolishes the decrease of PTEN phosphorylation induced by Lovastatin treatment, but
already by itself leads to an upregulation of p-PTEN level (Figure 5a). This phosphorylation change leads to reduced PTEN activity, confirming that blocking BMP has the opposite effect on PTEN as BMP treatment in breast cancer cells, as would be expected. To avoid cell line bias we repeated the essential experiments using two other CRC cell lines treated with Lovastatin. The colon cancer cell line RKO with intact canonical BMP signaling shows a strong reduction of mTOR phosphorylation after statin treatment. HT29 in contrast shows no effect. HT29 is SMAD4 deficient thereby inactivating canonical BMP signaling. This cell line is also resistant to Statin treatment in vitro and in vivo, as we have shown previously. (Figure 5b).

Figure 3
A. Kinase activity was deduced from the pattern of substrate phosphorylation using established algorithms and used to construct provisional signal transduction schemes to detail the effects of Lovastatin on colon cancer cell signal transduction. The numbers of the individual substrates on the array are shown in black and correspond to the numbers detailing the kinome profiling results in tables S1A and S1B
B. Immunoblotting using phosphorylation-specific antibodies was used to validate the kinome analysis and confirmed the results at protein level. In the array Statin treatment showed the strongest effect on the PI3K/Akt/mTOR pathway. Immunoblot analysis confirmed the downregulation of AKT, mTOR and its downstream target 70S6K.
Statin treatment leads to increased BMP signaling and downregulation of mTOR phosphorylation in xenografts

As statins are usually given orally to patients, we performed an in vivo experiment using xenografts of HCT116 cells in nude mice receiving three weeks of oral statin treatment before they were sacrificed and the tumors were analyzed. We feel this is an improvement on previous studies which have used intraperitoneal or intratumoral injection of statins to show effects on xenografts in mice. Effect of statin treatment on tumor size has been reported previously(16). In this study we were interested whether oral administration of statin would influence mTOR signaling in xenografts. Immunohistochemical analysis of nuclear pSMAD1,5,8 in the xenografts shows an significant increase in active BMP signaling in HCT116 xenografts (Figure 6 a,b), confirming our previous findings in vitro(16, 16).

Figure 4
A. Immunoblots for PTEN and p‐mTOR of HCT116 cells transiently transfected with either siRNA targeted against PTEN, or scrambled control siRNA and subsequently treated with Lovastatin or vehicle control. Actin was used as a loading control. Transfection with siRNA against PTEN leads to a significant knock-down of PTEN as shown by immunoblotting. SiRNA against PTEN leads to an increase in phosphorylated mTOR, but also diminishes the inhibitory effect of statin treatment on mTOR phosphorylation.

B. Immunoblots for AKT and p‐mTOR of HCT116 cells transiently transfected with either a constitutive active form of AKT (ca‐Akt), or control empty pcDNA and subsequently treated with Lovastatin or vehicle control. Actin was used as a loading control. Transfection with ca‐Akt leads to an increase in Akt protein and mTOR phosphorylation, but abrogates the inhibitory effect of Lovastatin treatment on mTOR phosphorylation.
17). As shown in Figure 6 (a, b) PTEN phosphorylation and mTOR phosphorylation are decreased in HCT116 colon cancer cell xenografts after statin treatment. Statin treatment leads to reduced pmTOR expression in vivo in humans. Data linking BMP and PTEN are mainly derived from in vitro studies using breast cancer cell lines and in vivo mouse studies in normal tissue or hamartomatous polyps (38, 40, 41). We therefore investigated whether treatment with statins would show similar results in vivo in humans. We approached patients newly diagnosed with CRC and treated them with 40mg Simvastatin orally once daily for three consecutive weeks. Biopsies taken at diagnosis (endoscopically) and tissue collected at surgical resection (after 3 weeks) were compared for BMP signaling activity measured by nuclear pSMAD1,5,8, and phospho-mTOR expression. Immunohistochemical analysis of the samples shows that treatment with the clinically relevant dose of 40mg Sim-

![Figure 5](image_url)

**Figure 5.**
A. Immunoblots for p-PTEN, p-mTOR and p70S6K of HCT116 cells treated with the BMP ligand inhibitor Noggin, prior to treatment with Lovastatin or vehicle control. Actin was used as a loading control. Noggin treatment alone leads to an increased in phosphorylation of PTEN and a slight increase in phosphorylation of mTOR and its downstream target p70S6K. The inhibitory effect of Lovastatin on PTEN and mTOR phosphorylation is abolished by Noggin treatment.
B. Immunoblots for p-PTEN and p-mTOR in RKO and HT29 cells with or without Lovastatin treatment. Actin was used as a loading control.
vastatin for 3 weeks leads to a significant decrease in phospho-mTOR expression (Figure 6 c, d). BMP signaling was not significantly increased despite showing a strong trend towards upregulation (Figure 6 c, d). Logistical and tissue handling problems meant that we were only able to perform a small pilot study. These preliminary results however suggest that the molecular effects of statins that we observe in vitro in colorectal cancer cell lines and in vivo in xenografts are also seen in vivo in humans.

**Figure 6.**
A. Immunohistochemistry for phospho-SMAD1,5,8, phospho-PTEN and phospho-mTOR of HCT116 xenografts from mice treated with Simvastatin or from control mice. The original magnification is 20x.
B. The percentage of cells with nuclear immunostaining for p-SMAD1,5,8, cytoplasmic immunostaining for phospho-PTEN and cytoplasmic immunostaining for phospho-mTOR relative to all cells per 20x image field in the HCT116 xenografts. Three images were scored for each xenograft. Error bars represent SEM (n=8).
C. Immunohistochemistry for phospho-SMAD1,5,8 and phospho-mTOR of human colon cancer biopsy samples taken at diagnosis and resection material of the colon cancers after patients were treated with Simvastatin 40mg for 3 weeks. The original magnification is 20x.
D. The percentage of cells with positive nuclear immunostaining for p-SMAD1,5,8, and positive cytoplasmic immunostaining for phospho-mTOR relative to all cells per 20x image field from the patient tissue samples. Three images were scored for each patient.
DISCUSSION

Genetic changes and the subsequent alterations in signal transduction pathways are key features of carcinogenesis(42) and new therapeutic strategies targeting these pathways have proved successful in treating CRC but have to be targeted to cancers with a sensitive molecular profile. This paradigm seems to be equally true for non-biological therapies such as Aspirin which only reduces the incidence of COX-2 overexpressing cancers, and cancers with PI3K3A mutations (43, 44). This underscores the importance of understanding the molecular mechanism of action of compounds that appear to have beneficial effects in CRC in epidemiological studies. In this study we set out to investigate the early effects of statin treatment on CRC cell signal transduction using a kinome array. We show that statins lead to most pronounced effects on the Akt/mTOR survival pathway via PTEN. Furthermore the effect of statin treatment on PTEN and mTOR is BMP dependent. For the kinome array analysis we chose to use the HCT116 colon cancer cell line, as we have previously shown HCT116 cells to be sensitive to statin treatment(16). The major effects on pmTOR level were also studied in RKO and HT29 cell lines and show that both cell lines show downregulation of phospho-mTOR after statin treatment. This shows that the effect of statins on mTOR pathway is not unique to HCT116 cells. Further work is needed to relate the sensitivity of individual cancers to mTOR inhibition to their mutational fingerprints(45). Whether statins work through the same mechanisms in cancers with different subtypes (CIN, CIMP, and MSI) also needs further investigation although the cell lines we have tested cover these essential subtypes.

Multiple studies have been performed to investigate the molecular mechanism of statins in cancer (18, 46). However, often data have been obtained using high concentrations of statins in vitro. Therefore, we decided to investigate which concentration of Lovastatin would be appropriate for studying specific effects on global cell signaling. The kinome array analysis shows that treating cells with high doses of statins (10µM) for 24 hours leads to chaotic changes in kinase activity which could represent non-specific toxic effects. Conversely, kinome analysis of cells treated with relative low dose of statins (2µM) that are achievable in human serum in vivo exhibit consistent changes within known signaling pathways, with the most profound effect being reduced activation of the Akt/mTOR pathway, a major survival pathway which is frequently upregulated in CRC (47). It has been shown that statins can inhibit the Akt/mTOR pathway in breast, lung, hepatic and renal carcinoma (48). Here we show using kinome and immunoblot analysis that this is also the case for CRC cells. We go on to show that siRNA knockdown of PTEN abolishes the inhibition of mTOR activity due to statin treatment, consistent with the effect of statins on mTOR being mediated through its effects on PTEN. Transfection with constitutively active Akt also prevents statins from inhibiting mTOR providing further evidence that mTOR inhibition by statins is PTEN dependent.
Studies have shown a link between PTEN expression and active BMP signaling in breast cancer cell lines and in mouse studies (38, 40, 41). We have previously shown that statins induce BMP signaling and induce apoptosis in CRC cells and this effect can be abolished by either Noggin or mevalonate treatment (16). In this study we therefore investigated whether the effect of statin treatment on PTEN was mediated through induction of BMP signaling. We show that when BMP signaling is inactivated due to pre-treatment with the specific BMP inhibitor Noggin, statins no longer have an inhibitory effect on mTOR. Noggin alone increases phosphorylated PTEN levels and also reduces the effect of statins on PTEN phosphorylation. This strengthens the link between BMP signaling and PTEN, and is consistent with the effect of statins on PTEN being mediated via effects on BMP signaling, although the exact mechanism of this interaction needs further investigation.

To confirm these results in vivo we studied xenografts in nude mice treated with Simvastatin and treated a small number of patients with colorectal cancer for 3 weeks with Simvastatin between diagnosis and surgical resection. We confirmed that statins activate canonical BMP signaling by demonstrating significant increases in nuclear pSMAD1,5,8. New is our finding that oral statin administration also leads to a significant decrease in PTEN and mTOR phosphorylation in the HCT116 xenografts. Some of these effects are also seen in a clinical setting in patients treated with orally administered Simvastatin for 3 weeks. In a small pilot study BMP signaling was not significantly upregulated, but showed a strong trend, while phospho-mTOR levels were significantly decreased in cancer specimens after treatment. Unfortunately, phospho-PTEN immunohistochemistry in the patient specimens revealed no staining in the resection specimens. This may be due to the relatively long warm ischemic time or differences in fixation compared to the xenografts. Phospho-epitopes are known to be very sensitive to delays in fixation (Ischemic time impacts biological integrity of phospho-proteins in PI3K/Akt, ERK/MAPK, and p38 MAPK signaling networks (49). The pilot patient study was smaller than intended. The study was stopped prematurely due to poor patient inclusion partly due to high levels of Statin and NSAID use and partly to delays in surgical resection in many included patients leading to protocol violations. A future larger patient trial will be needed to further confirm the results in patients.

In conclusion, statin treatment of CRC cells leads to widespread changes in kinase activity affecting multiple signaling pathways with the most pronounced effects on the PI3K/Akt/mTOR pathway and PTEN phosphorylation level which are BMP-dependent. Kinome analysis is a powerful, but relatively novel technique, which may miss some alternative mechanisms. However, the broad, hypothesis-free approach and the confirmation of the major effects in mice and pilot experiments in humans, provide new insights into the working mechanisms of statins which could facilitate their rational, targeted use in CRC.

Acknowledgements: Grant support: the Dutch Cancer Society (KWF)
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