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Chapter 3

Simvastatin in G13D KRAS mutated colorectal cancer cells render cells susceptible for cetuximab
Abstract

Introduction
Statins are commonly used to reduce cholesterol levels and lower the cardiovascular risk. Beside cholesterol, also the formation of farnesylpyrophosphate (a C15-group) and geranylgeranylpyrophosphate (a C17-group) are inhibited. These groups are used to activate the KRAS protein by prenylation (addition of a C15 or C17 group). After prenylation, KRAS becomes more lipophilic and translocates from the cytosol to the membrane. In this study, we hypothesized that the cetuximab resistant phenotype of KRAS mutant cancer cells could be converted to a more KRAS wild type phenotype rendering the cells susceptible for cetuximab, by co-incubating cells with simvastatin.

Method
Survival assays to measure proliferation were performed to study the effect of simvastatin, cetuximab and combination in the KRAS wild type A431 and KRAS mutant LoVo, HCT116 and SW480 cell lines. Furthermore KRAS localization assays as well as kinase activity assays were performed for all cell lines, to further explore the potential mechanisms.

Results
Simvastatin combined with cetuximab resulted in decreased proliferation in KRAS codon 13 mutated cell lines. Especially in the KRAS G13D mutant LoVo cells a synergistic effect on inhibition of proliferation by the combination treatment was observed. After incubation with simvastatin more KRAS protein was situated in the cytoplasm in KRAS mutant cells compared to control cells. The EGFR target pathway is controlled by tyrosine kinase activities, which showed synergistic inhibition in LoVo cells. Simvastatin elevated Serine/Threonine kinase activities, including AKT and NOS phosphosites, which is in accordance to previous reports.

Conclusion
In summary, we observed a synergistic effect of adding simvastatin to cetuximab treatment in inhibition of KRAS codon 13 mutated CRC cell proliferation. The mechanism of action appears to involve aberrant translocation of KRAS likely due to lack of prenylation and enhanced inhibition of tyrosine kinase signaling.
Effects of simvastatin and cetuximab in KRAS mutant colorectal cancer cells

Introduction

Epidermal growth factor receptor (EGFR) signaling plays an important role in proliferation of cells, and therefore blocking this pathway has emerged as an effective drug target in oncology. Cetuximab and panitumumab are two registered EGFR antibodies. Patients with advanced or metastasized colorectal carcinoma (CRC) failing fluorouracil (or alternatives such as capecitabine or uracil and tegafur, oxaliplatin and irinotecan) can be treated with chemotherapy combined with one of these anti-EGFR monoclonal antibodies. However, cetuximab and panitumumab have both been registered for the use in patients with KRAS wild type tumors only, since retrospective analyses of clinical trials showed a lack of efficacy in KRAS mutated colorectal tumors. Unfortunately, approximately 40% of patients with colorectal cancer have a somatic KRAS mutation, which leads to a constant expression of KRAS protein, operating independently of EGFR and thus EGFR antibody resistance. As a result, a considerable group of CRC patients is excluded from therapy with cetuximab or panitumumab and does not benefit from EGFR antibody treatment [1].

KRAS can be mutated at several positions, 90% of the activating KRAS mutations occur at codon 12 and 13[2]. Membrane association of the KRAS protein is crucial for its function as a switch in the signal transduction pathway between EGFR and the nucleus. To achieve this, the hydrophilic KRAS protein is farnesylated (addition of farnesylpyrophosphate, a farnesylgroup), or geranylgeranylated (addition of a geranylgeranylpyrophosphate, a geranylgeranylgroup). As a result, KRAS becomes more lipophilic and translocates from the cytosol to associate with the cell membrane. This so-called prenylated active KRAS exerts its function in the cellular membrane [3,4]. Statins are known to inhibit the conversion of 3-hydroxy-3-methyl-glutaryl Co-enzyme A (HMG-CoA) to mevalonate, a precursor for cholesterol synthesis; this reduces the cholesterol synthesis and thus the formation of low-density lipoprotein. Beyond their lipid lowering effects, statins are extensively studied for their effects on cellular proliferation in cancer. As shown in in vitro studies, simvastatin affects angiogenesis, apoptosis as well as the inflammation processes. [5-8] Another important effect of statins, although less studied, is the interference with the formation of farnesyl- and geranylgeranylgroups. These groups are formed as part of the mevalonate cascade and are crucial for the prenylation of proteins, such as KRAS. Since farnesyl- and geranylgeranyl moieties are essential for post-translational prenylation, and thus for activation of KRAS. Statins may have the potential to phenoconvert KRAS mutated tumors into a more KRAS wild type and thus EGFR inhibitor sensitive phenotype [9,10].

In this proof of concept study we hypothesized that phenoconversion of KRAS mutant colorectal cancer cells could be achieved by simvastatin, rendering colorectal cancer cells sensitive for cetuximab. In this study, cell growth survival assays were used to investigate a possible synergistic effect of simvastatin on cetuximab sensitivity in several KRAS mutant colorectal cancer cell lines and in wild type EGFR overexpressing cell line. The effect of simvastatin on membrane association of KRAS was investigated, and in addition, overall kinase activity upon treatment was explored.
Material and Methods

Cell lines
The human colorectal cancer cell lines LoVo (KRAS mutation G13D), HCT116 (KRAS mutation G13D), SW480 (KRAS mutation G12V), and the human epidermoid carcinoma cell line A431 (wild type for KRAS and over-expressing EGFR) were obtained from American Type Culture Collection-ATCC (Manassas VA, USA). These cell lines were selected using the information about KRAS mutations in the Cancer Genome Project (http://www.sanger.ac.uk/genetics/CGP/CellLines/). Cell lines harbouring a KRAS mutation in codon 12 or 13 were selected because these mutations are most common in colorectal cancer. Cells were grown in RPMI 1640 medium (Invitrogen, Breda, The Netherlands) supplemented with 10% fetal bovine serum (Greiner Bio-One GmbH, Frickenhausen, Germany) and 1% penicillin/streptomycin (Invitrogen, Breda, The Netherlands) in a 5% CO2 atmosphere at 37°C. When applicable, cells were detached from flasks with Trypsin-EDTA solution (Invitrogen, Breda, the Netherlands). Cells were cultured for a maximum of 20 passages. Cetuximab was kindly provided by Merck (Darmstadt, Germany). Simvastatin was obtained from Fagron (Nieuwerkerk a/d IJssel, The Netherlands) and was chemically activated by alkaline hydrolysis prior to use as described before [11]. Simvastatin was selected because this statin is most commonly prescribed in Europe.

Cell survival
The effects of simvastatin and cetuximab as single agents or in combination on survival of the cells, were evaluated using the sulphorhodamine binding (SRB) colorimetric assay as described by Skehan et al. [12] with minor modifications. Cells were harvested by trypsinization, counted and plated in 96-well plates at a density of 5 x 10^3 cells per well (100 µL/well). Following overnight incubation, cells were pre-treated with simvastatin (2 µM). After 24 hours of incubation, cells were co-treated with cetuximab (500 µg/ml) and incubated for another 48 hours. At the end of the incubation, the SRB assay was performed as described below. Cells were fixed by addition of 25 µl ice-cold 50% trichloroacetic acid (TCA) to the growth medium. The plate was incubated at 4°C for 1 hour and then the cells were gently washed three times with milli-Q water. After drying at room temperature, cells were stained with 50 µl of 4% sulphorhodamine (w:v dissolved in 1% acetic acid) for 30 min. At the end of the staining period, unbound sulphorhodamine was removed by washing three times with 1% acetic acid. The plates were air dried and bound SRB was dissolved in 200 µl of 10 mM Tris-base (pH 10.5). Next, the plate was shaken followed by reading the optical density (OD) at 550 nm in a microplate spectrophotometer (Spectramax 190, Molecular Devices, Sunnyvale, USA). Results are expressed as the relative percentages of absorbance compared to controls, which were not exposed to drugs. Results of OD measurements are expressed as a percentage of cell proliferation of the controls. Results are expressed as means with corresponding standard deviations of three independent experiments in at least fourfold. Statistical analysis was performed using SPSS, a two-sided t-test were performed and p-values <0.05 were considered significant.

Membrane association of KRAS
Cell were seeded in a 6 wells plate at a density of 5 x 10^3 cells per well. After overnight incubation, cells were incubated for 24 hours according to the following conditions: vehicle as negative control, 2 µM simvastatin or with 2 µM of the geranylgeranyltransferase inhibitor GGTI-298 (Merck, Darmstadt, Germany). GGTI-298 is known to inhibit prenylation and therefore taken
as positive control. Next, cells were trypsinized and membrane and cytoplasm fractions were separated using Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Scientific, Breda, The Netherlands). Protein concentration was determined using BCA protein assay kit (Thermo Scientific, Breda, The Netherlands). Presence of KRAS protein in membrane and cytoplasm fractions was analysed by western blotting. Equal amounts of cell lysates were loaded on 11% SDS-PAGE gels. After blotting on nitrocellulose membrane, the blot was cut in two to detect KRAS and Actin (as internal control) in the same lane. Primary antibodies against KRAS (RAS (D2C1), Cell Signaling Technology, Leiden, The Netherlands) and Actin (actin (13E5), Cell Signaling Technology, Leiden, The Netherlands)) were used in this study. Anti-rabbit IgG, HRP-linked antibody and LumiGlo (Cell Signaling Technology, Leiden, The Netherlands) was used to visualize the proteins bands on Chemidoc from Biorad (Veenendaal, the Netherlands).

**Kinase activity profiling**

Cells, counted and plated in 6-wells plates at a density of 1 x 10^6 cells per well (2 mL/well) were harvested by trypsinization. Following overnight incubation, cells were pre-treated with 2.0 μM simvastatin or vehicle for 24 hours and another 24 hours with wicicle, simvastatin, cetucimab or combination. At the end of the incubation, cells were lysed using M-PER Lysis buffer (Fischer Thermo Scientific, Rockford, USA) supplemented with Protease inhibitor Cocktail and phosphatase Inhibitor cocktail (Fischer Thermo Scientific, Rockford, USA) (1 million cells per 100 µl). The protein content was determined with Pierce BCA Protein Assay Kit (Thermo Scientific, Breda, The Netherlands). Protein serine/threonine kinase activity and tyrosine kinase activity was determined in triplicate using Pamgene's Serine/Threonine Kinase (STK) peptide or tyrosine Kinase (PTK) microarrays (Pamgene, ’s-Hertogenbosch, the Netherlands), according to the manufacturers’ instructions [13]. All microarray data processing and visualizations were performed using Bionavigator and Matlab software (R2010B, The Mathworks, Natick, MA) as described before[14]. For the serine/threonine kinase assay 109 peptides were included in the analysis, for the PTK assay, 89 peptides were included and analysed in the R-package 'multcomp' using Dunnett contrasts. For pathway analysis, substrate peptides that showed a significantly different (p<0.05) phosphorylation pattern between vehicle and simvastatin, cetuximab or combination were used as input to identify pathways with differential activity using GeneGo Metacore (Thomson Reuters). The peptides were linked to the UniProt ID's of the proteins that they were derived from Uni-Prot database (www.expasy.org) and these were used for a pathway analysis.

**Results**

**Effect of simvastatin and cetuximab on survival of KRAS mutant and wild type cell lines**

The cytotoxicity of simvastatin was first tested in a concentration range from 0.1 to 2.0 μM (data not shown). A minimal cytotoxic effect was observed and therefore a concentration of 2.0 μM of simvastatin was used in the experiments to inhibit the mevalonate pathway and the formation of farnesyl- and geranylgeranylgroups. Incubation of the KRAS wild type A431 cell line with 500 μg/ml cetuximab resulted in a decrease of survival to 66 ± 3.3 % compared to untreated cells (p=0.045) (figure 1). The combination of both simvastatin and cetuximab diminished cell growth further to 56 ± 4.1 %, but this was not significantly different from the growth inhibitory effect of cetuximab as single agent (p=0.228). Incubation of LoVo and HCT116 cells, both
harbouring a KRAS G13D mutation, with 500 µg/ml cetuximab resulted in a non-significant survival reduction of 84 ± 11.1 % and 99 ± 5.5 %, respectively compared to control. In these cell lines, compared to control, simvastatin monotherapy (2 µM) resulted in a survival of 76 ± 9.0 % for LoVo and 84 ± 7.5 % for HCT116. Combined treatment with simvastatin and cetuximab reduced the proliferation in LoVo further to 47 ± 4.9 %, which is significantly different from the growth inhibitory effect of either cetuximab (p<0.001) or simvastatin (p<0.001) as single agents. In HCT116 the same synergistic effect was seen, combination therapy significantly reduced the survival to 68 ± 5.6 % compared to simvastatin alone (p<0.002) or cetuximab alone (p<0.001). No synergistic effect was observed in SW480 cells (KRAS G12V) when treated with simvastatin and cetuximab as well. Incubation with 500 µg/ml cetuximab did not result in a decrease of survival (102 ± 1.7 % p=1.00). Simvastatin (2 µM) as single agent resulted in survival of 79 ± 7.5%. Compared to treatment with simvastatin alone, the combination with cetuximab, did not result in an additional reduction in the survival of the SW480 cells (73 ± 10.9 %).

Figure 1: Cell proliferation assays (Sulforhodamine-B assays) of three KRAS mutant (LoVo, HCT 116 and SW480) and one KRAS wild type cell line treated with simvastatin, cetuximab or the combination for three days. The results of the measured optical density are expressed as a percentage of cell proliferation compared to the untreated controls. Results are means and the 95 % confidence intervals of three independent experiments in at least fourfold.
**KRAS translocation**

To test whether the observed synergistic effect was the result of decreased KRAS activation, the effect of simvastatin on the prenylation of mutated KRAS was analysed in LoVo and HCT116 cells. Membrane and cytoplasm fractions of cells incubated with simvastatin were tested for KRAS localization. In both cell lines, KRAS was mainly localized in membranes of the cell. Upon simvastatin treatment, an increase of KRAS concentrations in the cytoplasm of LoVo and HCT116 cells was observed (figure 2a and 2b). This was also seen in presence of the positive control GGTI-298 (Sigma-Aldrich, the Netherlands) an established prenylation inhibitor.

**Figure 2 a and b:** Western blot analysis showing the amount of KRAS in the cytoplasm and membrane after treatment with the positive control GGTI-298 or simvastatin in KRAS mutant G13D HCT116 cells (a) and LoVo cells (b).
**Kinase activity profiling**

As described above, a clear synergistic effect of simvastatin on cetuximab treatment was observed in LoVo cells. Therefore, PTK and STK activity was explored for these cells at different treatment conditions. STK activity reflects downstream signaling of KRAS. In LoVo cells, treatment with cetuximab or simvastatin as single agents display a strong activated STK activity, nearly all peptides on the STK array became significantly phosphorylated. Because of this strong effect, no conclusion could be drawn. PTK activity reflects EGFR signaling, upstream of KRAS. Cetuximab and simvastatin as single agents showed only a slight effect on PTK activity, however, combination of both drugs show a clear synergistic effect displayed by decreased PTK activity (figure 3A and 3B). In all other cell lines (A431, HCT116 and SW480), this synergy was not observed.

**Figure 3a:** STK activity profiles. Significant (p < 0.05) log ratio of signal intensity versus the control sample for cell lines A431, HCT116 and LoVo, treated with simvastatin, cetuximab or combination. Each column represents a sample, each row a peptide. The relative signal with respect to control is indicated by the color intensity: red implies higher, blue lower than control.
Discussion

In this study we investigated if phenoconversion of KRAS mutant colorectal cancer cells by simvastatin could be achieved in order to restore sensitivity of KRAS mutant cells to anti-EGFR treatment. We also explored overall kinase activity upon treatment. Our study shows that simvastatin affects KRAS localization and that simvastatin treatment of cells results in decreased membrane association of KRAS. Furthermore, KRAS codon 13 mutant cells become sensitive for cetuximab in presence of simvastatin. Exploring kinase activity revealed a decrease in PTK activity in LoVo cells upon treatment with simvastatin together with cetuximab, explaining the significant decrease in cell survival.

The proliferation of KRAS G13D mutated LoVo and HCT116 cell lines was significantly inhibited in presence of both drugs, whereas the individual drugs have no or only a minimal effect on cell proliferation. However, in the SW480 cell line, harbouring a KRAS codon 12 mutation the combined therapy of cetuximab and simvastatin did not significantly affect the proliferation. This indicates that not all KRAS mutations in colorectal cancer cells have a similar effect on anti-EGFR sensitivity, although other mutations should also be taken into account. In a recent study computational analysis revealed that the codon 13 mutated KRAS protein has a similar structure compared to the wild type protein [15]. This similar structure of KRAS may contribute to difference in effect between KRAS 12 and 13 mutations observed in this study. Also retrospective analysis in...
patients showed that patients with G13D mutant colorectal tumor might respond to cetuximab treatment[16,17]. Our findings are in agreement with the preclinical study of Lee et al. [18] who also showed that simvastatin might overcome cetuximab resistance in colorectal cancer by modulating BRAF activity, an important kinase in the RAS-RAF–MAPK signaling pathway[19]. Other studies showed that simvastatin also affects angiogenesis, apoptosis and the inflammation processes [5-8] but at higher concentrations (up to 50 μM). In our experiments a low simvastatin concentration of 2 μM was used, aiming at affecting only the prenylation of KRAS without a direct cytotoxic effect. Of note, KRAS mutant cells are more susceptible simvastatin than the wild type KRAS cell line. A possible explanation for this is a strong dependence (addiction) of the mutant cells on permanently activated KRAS and its corresponding pathways. A recent preclinical study [20] showed that simvastatin indeed inhibited the geranylgeranylation of the KRAS protein and this inhibition could be reversed with addition of geranylgeranylpyrophosphate. The geranylgeranyltransferase inhibitor GGTI-298 was used as a positive control in the Westernblot experiments. Incubation with this inhibitor indeed resulted in elevated levels of KRAS in the cytoplasm. Western blot analysis used here showed that membrane association of mutant KRAS is inhibited by simvastatin in the KRAS G13D cell lines LoVo and HCT116. This effect is most likely caused by a decrease in prenylation of the KRAS protein after simvastatin treatment.

The phosphorylation pattern was clearly affected by simvastatin in the KRAS mutated cell lines. Whereas this effect was absent in the wild type cell line. The differences in kinome profiles after simvastatin treatment also supports the aforementioned dependence on KRAS signaling in KRAS mutant cell lines. The results from the kinome analysis showed that simvastatin altered the KRAS dependant signaling in the cancer cell lines, especially in KRAS codon 13 mutated cells. Pamchip STK data revealed, most clearly seen in LoVo cells, an abundant STK activity (of which PKB/AKT). These findings are in accordance with the findings of Kureishi et al.[21]. Skalez-Rorowski[22] showed that low doses of statins promote AKT activation while high doses result in toxicity and cell death[23]. Because of the tremendous STK activity detected in LoVo cells, more subtle effects downstream of KRAS could not be observed by the Pamchip array. However, the observed synergistic effect of simvastatin and cetuximab on KRAS codon 13 mutated cells, could well be explained by Pamchip PTK data. EGFR is a tyrosine kinase receptor of which signaling could be blocked by cetuximab. As expected, LoVo cells show a decreased PTK activity upon incubation with cetuximab. Hardly any effect was seen upon simvastatin treatment, also as expected since simvastatin was considered to affect only STKs. Interestingly, the synergistic effect of both drugs on cell survival was also seen on PTK activity. As mentioned before, conform the results of Lee et al [24] simvastatin and cetuximab synergise after BRAF (effector protein downstream of KRAS) modulation. A recent report by Prahallad et al. [25] showed that blocking mutated BRAF by vemurafenib resulted in feedback activation of EGFR. Indeed, these cells became sensitive for cetuximab. Taken together, their and our findings, interfering in the signaling pathway downstream of KRAS (by simvastatin or vemurafenib), results in feedback activation of EGFR. Consequently, PTK activity is diminished upon treatment by both drugs.

In conclusion, the cetuximab resistant phenotype of KRAS codon 13 mutated cell lines, could be converted to a more KRAS wild type cell line (which is moderately sensitive to cetuximab) by co-treatment with simvastatin. The mechanism behind this phenoconversion is interfering in membrane association of KRAS most likely by inhibition of prenylation of KRAS. Interfering in the pathway downstream of KRAS results in a feedback mechanism of EGFR, resulting in a synergistic effect of simvastatin on cetuximab treatment. The latter conclusion is confirmed by the observed decreased PTK activity.
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References


Chapter 3

Chemother Pharmacol 64: 657-663.


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