Additive antitumor effects of celecoxib and simvastatin on head and neck squamous cell carcinoma \textit{in vitro}

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Abstract. Lipid-lowering statins as well as non-steroidal anti-inflammatory drugs (NSAIDs) have been reported to possess cancer-protective effects in many epidemiologic cohort studies. However, the underlying mechanisms of these findings are mostly unknown. To evaluate possible additive antitumor effects of statins and NSAIDs \textit{in vitro}, PJ-41 and HLaC78 head and neck squamous cell carcinoma cells (HNSCC) were treated with 40 \(\mu\)M celecoxib, 50 \(\mu\)M simvastatin or a combination of both. Analysis of tumor viability, proliferation, apoptosis, cell cycle changes and secretion of interleukin-6 (IL-6) and interleukin-8 (IL-8) was conducted via MTT assay, Annexin V-propidium iodide test, cell cycle analysis, colony assay and enzyme-linked immunosorbent assay (ELISA). Celecoxib and simvastatin alone as well as a combined treatment showed a significant reduction in tumor cell viability, proliferation and secretion of IL-6 and IL-8 compared to the control group. The combined treatment even proved to have significantly greater effects. We postulate that simvastatin and celecoxib have additive antitumor effects on HNSCC \textit{in vitro}, which warrants further investigation.

Introduction

Squamous cell carcinoma of the head and neck (HNSCC) is one of the most commonly occurring malignancies, and is a major cause of cancer morbidity and mortality worldwide with an estimated incidence of 500,000 per year in the USA (1). The overall 5-year survival rate for pharyngeal and oral squamous cell carcinoma is approximately 60% in the USA, and has not changed significantly during the past 40 years (2). Due to the increasing spread of the human papillomavirus within the oropharyngeal tract, which represents a new pathogenic factor, the incidence of HNSCC is estimated to rise even more (3). Thus, there is a tremendous need for new treatment options for HNSCC patients.

Statins are widely used as cholesterol-lowering drugs, being small-molecule inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (4). In addition to their common use in the treatment of lipid disorders, statins have also demonstrated anticarcinogenic properties in various preclinical \textit{in vitro} studies (5,6). This has been attributed mainly to the inhibition of isoprenoid and cholesterol synthesis, which are both important processes in the intracellular signaling pathways (6,7). Several observational human studies have reported a potential beneficial effect of statin use against the overall risk of cancer (8-10). Other studies, however, reported no such protective effects (11,12). For several specific cancers, especially for colorectal cancer (13), lung cancer (14,15) and renal cell carcinoma (16), protective effects of statin use have been published.

NSAIDs have also been demonstrated to have a potential chemoprotective effect. This has been explained by an induction of cell cycle arrest in G1-Phase via inhibition of Akt (17), inhibition of Ca\(^{2+}\) ATPase activity (18) or activating p53 and p21 (19). Several studies have reported on a protective effect of Aspirin on colorectal adenoms as well as colorectal carcinomas, even in randomized, double-blinded and placebo-controlled study designs (20-23). For selective cyclooxygenase (COX)-2 inhibitors such as celecoxib or rofecoxib, protective effects could also be demonstrated, again mainly for colorectal carcinoma (24-26). However, since there have been serious cardiovascular complications regarding long-term therapy with COX-2-inhibitors (27-29), the relatively high doses needed for the observed cancer-protective effect are being questioned. Therefore, a combination with other drugs with synergistic effects to reduce the dosage of NSAIDs is warranted.

A combined therapy of statins and NSAIDs has already been demonstrated to have a synergistic effect on the induction of apoptosis in prostate and colorectal cancer cells \textit{in vitro} (30,31). \textit{In vivo}, a low-dose combination of atorvastatin and celecoxib was reported to have synergistic antitumor effects on colorectal cancer in a xenograft animal model (32). For colorectal cancer, population-based studies have also shown synergistic cancer-protective effects of a combination...
therapy with statins and NSAIDs compared to the respective monotherapies (33,34). Yet for other entities, for example squamous cell carcinoma, few studies have been conducted to date.

The objective of the present study is to analyze the possible additive effects of the combined use of simvastatin and celecoxib on human HNSCC cells in vitro in terms of viability, cell growth, apoptosis and cell cycle changes. Additionally, secretion of selected interleukins, namely IL-6 and IL-8, was analyzed. IL-6 plays a key role in cell proliferation, apoptosis and differentiation. IL-6 induces activation of Janus kinase 1/2 (JAK1/2), resulting in phosphorylation of STAT3 at tyrosine-705 (Y705) (35). Activation of IL-6/STAT3 signaling has a significant role in self-renewal and acquisition of malignant features of cancer stem cells (CSC) (36). Furthermore, IL-6 levels are highly elevated in metastatic diseases and increased levels of serum IL-6 are associated with poor disease outcome and prognosis in human cancers (37,38). IL-8 is reported to be related to different malignancies due to the involvement of thrombophilia and angiogenesis (39). Increased secretion of IL-8 has been shown to increase the metastatic ability of different cancer entities (40,41). Therefore, analysis of IL-6 and IL-8 was included into the present study.

Materials and methods

Cell culture. The head and neck squamous carcinoma cell lines PE/CA-PJ 41 and HLaC78 were obtained from ECACC (European Collection of Cell Cultures, Salisbury, Wiltshire, UK). Cells were grown in rPMI-expansion medium (Biochrom AG, Berlin, Germany) with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% sodium pyruvate (100 mM, Biochrom AG), and 1% non-essential amino acids (100-fold concentration, Biochrom AG). Cells were cultured at 37°C with 5% CO₂ in culture flasks. Medium was replaced every other day and passaging was performed after reaching 70-80% confluence by trypsinization, with subsequent washing and seeding in new flasks or treatment wells. Experiments were performed using cells in the exponential growth phase.

Exposure to celecoxib and simvastatin. The half-maximal inhibitory concentrations (IC₅₀) of celecoxib (Pfizer Pharma PFE, Berlin, Germany) and simvastatin (MIP Pharma, Blieskastel, Germany) on PE/CA-PJ 41 were evaluated with the MTT assay (Fig. 1). To this end, PE/CA-PJ-41 cells were treated with 40 µM/ml celecoxib, 50 µM/ml simvastatin or the combination of both. Analytical assays were performed after 24 h of incubation.

\[3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.\] After 3 days of co-culture, the MTT assay (Sigma-Aldrich, Taufkirchen, Germany) was performed according to Mosmann (42) to determine cell viability. Cells were seeded at 10,000 cells per well in a 12-well plate. All wells were incubated with 1 ml MTT (1 mg/ml) for 5 h at 37°C and 5% CO₂. MTT was then removed and 1 ml isopropanol was added, followed by another incubation period of 1 h at 37°C and 5% CO₂. Measurement of the color conversion of the blue formazan dye was performed using a multi-plate reader (Titertek Multiskan PLUS MK II; Thermo Labsystems, Thermo Fisher Scientific, Inc., Darmstadt, Germany) at a wavelength of 570 nm.

Colony assay. PE/CA-PJ 41 were seeded into 6-well plates at a concentration of 2,5x10⁵ cells/well in triplicate. Celecoxib (40 µM), 50 µM simvastatin or the combination of both were added to defined well plates. PE/CA-PJ 41 cultivated in RPMI-EM served as the control. Cells were incubated for 14 days. After 2 weeks the well plates were stained with crystal violet, and colonies were counted manually.

Annexin V-propidium iodide test. The BD Pharmingen Annexin V-APC kit (BD Biosciences, Heidelberg, Germany) was used to evaluate apoptosis on HLaC78 and PE/CA-PJ 41. After 3 days of co-culture, cells in suspension and adherent cells were harvested, then washed twice with PBS and resuspended in 1:10 binding buffer [0.1 M HEPES (Sigma-Aldrich) (pH 7.4), 1.4 M NaCl, 25 mM CaCl₂] at a concentration of 1x10⁶ cells/ml. Aliquots of this cell suspension (100 µl; 1x10⁵ cells) were then transferred to a 5 ml culture tube. Propidium iodide (5 µl) and Annexin V-APC (5 µl) were added to each aliquot. After 15 min of incubation at room temperature in the dark, the cells were resuspended with 400 µl 1:10 binding buffer. A FACSCanto flow cytometer was used to analyze the samples with BD FACSDiva version 5.0.3 software (BD Biosciences). Only cells with damaged membranes were stained by propidium iodide.

Cell cycle analysis. To analyze the effect of celecoxib and salinomycin on the cell cycle of PE/CA-PJ 41 and HLaC78, 1x10⁶ cells were cultivated in 12-well plates in triplicate. Following a 48 h period, PE/CA-PJ 41 cells were trypsinized and washed twice with cold PBS. Cells were then fixed in 1ml of 70% cold ethanol in test tubes and incubated for 2 h at 4°C in the dark. After incubation, cells were centrifuged at 500 x g for 5 min at 4°C and resuspended in 500 µl propidium iodide (BD Bioscience). After another incubation at 4°C in the dark

Figure 1. Evaluation of IC₅₀ for celecoxib (upper graphic) and simvastatin (lower graphic) using the MTT assay. Concentrations were identified as 40 µM for celecoxib and 50 µM for simvastatin (highlighted with asterisk and black column).

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for 15 min, cells were analyzed with flow cytometry within 1 h. PE/CA-PJ-41 cultivated in RPMI-EM served as the control.

**IL-6/IL-8 ELISA.** For measurement of the secretion of IL-6 and IL-8, the supernatants were collected (centrifugation, 150 x g for 5 min at 37°C) after 3 days of co-culture and stored at -20°C in sterile tubes until further use. RPMI-EM served as the control. Human IL-6 and IL-8 kits (catalog nos. 950.030.192 and 950.050.192, respectively; Diaclone SAS, Besançon, France) were used and the experiments were performed in duplicate. The ELISA plate was read at 450 nm (Titertek Multiskan PLUS Mk II). The concentrations of IL-6 and IL-8 were determined by constructing a standard curve using recombinant IL-6 and IL-8.

**Statistical analysis.** The data collected was transferred to standard spreadsheets and statistically analyzed using GraphPad Prism software (version 4.0; GraphPad Software, Inc., San Diego, CA, USA). Data are presented as the mean ± standard deviation of three experiments, unless otherwise stated. The Gaussian distribution was tested via first column analysis. One-way analysis of variance followed by Tukey's multiple comparison test was used. Additionally, multiplicity adjusted p-values were determined. p<0.05 was used to indicate a statistically significant difference. The combination index (CI) was applied to evaluate the interaction between celecoxib and simvastatin for PE/CA-PJ 41. CI analysis provides qualitative information on the nature of drug interaction, and the CI index, a numerical value calculated as described below, also provides a quantitative measure of the extent of drug interaction (43).

\[ CI = \frac{C_{A,X}}{IC_{X,A} + C_{B,X}} : IC_{X,B} \]

\( C_{A,X} \) and \( C_{B,X} \) are the concentrations of drug A and drug B used in combination to achieve x% drug effect (IC\(_{75}\), IC\(_{50}\)). IC\(_{X,A}\) and IC\(_{X,B}\) are the concentrations required for single agents to achieve the same effect. A CI of <0.85 was deemed to indicate synergy, a CI of >1.15 was deemed to indicate antagonism. Additive effects were assumed at an CI between 0.85 and 1.15.

**Results**

**MTT assay.** Viability of PE/CA-PJ-41 cells was analyzed using the MTT assay (Fig. 2). It revealed a significantly lower viability after addition of celecoxib, simvastatin and the combination of both compared to the control group (p<0.05 for all three). The combination of treatment with celecoxib and simvastatin also proved to decrease tumor cell viability significantly more compared to celecoxib and simvastatin alone (p<0.05 for both). Between treatment with celecoxib and simvastatin, no significant difference was found (p>0.05). The CI was calculated as 1.13, indicating a moderate additive effect (Fig. 3).

**Colony assay.** Tumor cell proliferation was analyzed using a colony assay (Figs. 4 and 5). Treatment with celecoxib, simvastatin and the combination of both showed significantly reduced cell colonies compared to the control group (p<0.05 for all three). Colony forming was higher when incubated with celecoxib alone versus simvastatin alone (p<0.05). The combination of both drugs showed no significant difference compared to celecoxib (p>0.05) or simvastatin (p>0.05) alone.

![Graph showing MTT assay results](image)

**Figure 2.** Evaluation of viability using the MTT assay. Lower viability of all three medication groups compared to the control (\(p<0.0001\)). Cross indicates a significant difference of combination therapy compared to both monotherapies (\(p<0.0001\)).

![Image](image)

**Figure 4.** Dishes used in the colony assay after staining 14 days after seeding. Compared to the control, celecoxib alone shows fewer cell colonies, while simvastatin alone and the combination of both have almost no colony formation.
Annexin V-propidium iodide test. Annexin V-propidium iodide analysis of PE/CA-PJ 41 (Fig. 6A) and HLaC78 (Fig. 6B) revealed enhanced apoptosis and necrosis after simvastatin treatment alone and after the combination of both compared to the control group (p<0.05 for both). The combination of both drugs induced higher rates of apoptosis and necrosis compared to simvastatin and celecoxib alone (p<0.05). Addition of celecoxib alone had no significant difference on apoptosis or necrosis compared to the control (p>0.05).

Cell cycle analysis. For both cell lines used, cell cycle analysis showed a significant increase in cells in G0/G1-phase when treated with celecoxib and simvastatin in combination compared to the control group (p<0.05), while celecoxib (p>0.05) and simvastatin (p>0.05) alone had no significant effect. The effect of the combination therapy was also significant compared to celecoxib (p<0.05) and simvastatin (p<0.05) alone (Fig. 7A and B).

Quantitative analysis of IL-6. Treatment with celecoxib, simvastatin and the combination of both all showed a lower secretion of IL-6 compared to the control group (p<0.05 for all three). Addition of simvastatin proved to decrease IL-6 secretion significantly more than celecoxib (p<0.05). The combined treatment of both drugs in turn showed less IL-6 than celecoxib (p<0.05) or simvastatin alone (p<0.05; Fig. 8).

Quantitative analysis of IL-8. Treatment with celecoxib, simvastatin and the combination of both all revealed a lower secretion of IL-8 compared to the control group (p<0.05 for all three). Incubation with celecoxib showed significantly
decreased IL-8 secretion than with simvastatin (p<0.05). The combined treatment of both drugs proved to reduce IL-8-production significantly more than celecoxib (p<0.05) or simvastatin alone (p<0.05; Fig. 9).

Discussion

Despite many advances in the therapy of HNSCC, survival rates remain low (44). Anticancer drug treatment for HNSCC today is mostly reserved for palliative chemotherapy regimens, which include cytostatic agents such as cisplatin, 5-FU or docetaxel as well as monoclonal antibodies such as cetuximab. However, these drugs offer small benefit with respect to progression-free survival, while in turn inducing severe side effects further limiting the use in cancer patients (45). Therefore, research for identifying new treatment options with reduced toxicities is warranted.

Simvastatin is an inhibitor of HMG-CoA reductase, an enzyme of the mevalonate synthesis pathway, which in turn inhibits formation of downstream lipid isoprenoids such as farnesyl pyrophosphate (FFP) and geranylgeranyl pyrophosphate (GGPP) (46). This in turn results in the side effect of decreased IL-8 secretion and diminished IL-6 secretion. The combined treatment of both drugs was significantly more effective than either drug alone, indicating a synergistic effect.

Figure 8. IL-6-ELISA. All three medication groups showed lower IL-6 levels than the control (**p<0.0001). Simvastatin showed decreased IL-6 levels compared to celecoxib (p<0.0001). The combination of both also showed lower IL-6 secretion than celecoxib or simvastatin alone (p<0.0001 and p=0.0144, respectively).

Figure 9. IL-8-ELISA. All three medication groups showed lower IL-8 levels than the control (**p<0.0001). Celecoxib showed decreased IL-8 levels compared to simvastatin (p<0.0001). The combination of both also showed lower IL-8 secretion than celecoxib or simvastatin alone (p<0.0001 for both).
decreasing cell proliferation via inhibition of Ras onco-
genesis (47). Statins have also been shown to induce apoptosis, reduce serum-stimulated Ras activity and increase messenger RNA (mRNA) and protein expression of the proapoptotic proteins Bax and Bad in esophageal carcinoma cell lines (48).

NSAIDs in general (21-23), as well as selective COX-2-
inhibitors (24-26), have already proven to be potent tumor-protective substances in vitro and in vivo. However, relatively high doses of NSAIDs or selective COX-2-inhibitors are needed to achieve the desired effects, which causes problems for long-term therapy due to the cardiovascular risks of these drugs (27-29). Thus, a combination of NSAIDs with other possibly synergistic drugs, for example statins, could be a solution for reducing the required doses for each.

The combination of statins and NSAIDs has already been demonstrated to have synergistic effects on colorectal cancer cells in vitro (30,31) and in an animal model in vivo (32). Yet, as of now few studies have evaluated the effects of this combination therapy on HNSCC cells. Thus, the present study focused on the synergistic effects of celecoxib and simvastatin on HNSCC cells in vitro.

The analyses showed significant reduction in PE/CA-PJ-41 tumor cell proliferation and viability after addition of celecoxib or simvastatin alone, with the effect increasing even more using a combination of both substances. This confirms results of colorectal cancer cells and prostate cancer cells treated with celecoxib and simvastatin in combination (30-32,46). The underlying mechanisms of these anticarcinogenic effects are not completely understood, however.

In the present study, these antitumor effects were mainly caused by apoptosis and, to a much lesser extent, by necrosis. By inhibition of HMG-CoA reductase, statins inhibit the synthesis of isoprenoids essential for membrane localization and subsequent activation of signaling proteins such as Ras, Rho and Rac, leading to increased apoptosis (4). Moreover, the reduction of cholesterol synthesis via statins and their inhibition of the Akt pathway has been shown to promote apoptosis in cancer cells (7). NSAIDs, on the other hand, also have a variety of possible mechanisms that determine their anticarcinogenic properties. Besides inhibition of Ca\(^{2+}\) ATPase activity (18), increase in ceramid levels (49), and inhibition of transcription activity of NFkB (50), NSAIDs have also shown the potential for inhibition of the Akt pathway, as does simvastatin (17). Even in concentrations that do not induce direct inhibition of Akt by celecoxib itself, it could be demonstrated that celecoxib significantly synergized atorvastatin to inhibit Akt-phosphorylation, indicating a pivotal synergistic effect of both substances regarding Akt-pathway-induced apoptosis (46).

In addition, significant cell cycle arrest in G0/G1-phase could be demonstrated for the combination therapy in the present study. Celecoxib has already been shown to induce cell cycle arrest at G1-phase via increased expression of cyclin-dependent kinase (CDK) inhibitors for various tumor cell types (51,52). The combination of atorvastatin and cele-
coxib has also been demonstrated to cause cell cycle arrest at G0/G1-phase at a significantly higher level than both substances alone (46). Thus, the induction of cell cycle arrest at G0/G1-phase could also be a potential synergistic effect. Since G0/G1-arrest inhibits the proliferation of tumor cells, it is a vital target for anticancer therapeutics. However, whether this G0/G1-arrest is irreversible, as has been described for Terfenadine (53), or perhaps even reversible, remains unclear since the present study only measured one time point after treatment. Preliminary analysis hint at an increase of cyclin-
dependent kinase inhibitors p21\(^{CIP1/WAF1}\) and p27\(^{KIP1}\) as a possible mechanism behind the G0/G1-arrest. However, in the present study no complete analysis of cell cycle protein expression has been conducted, limiting the information in this regard. It will be part of a future investigation at our institution.

The present study also revealed a significantly lower secretion of IL-6 and IL-8 by the tumor cells after addition of simvastatin and celecoxib combined rather than alone. IL-6 is a cytokine which, among other functions, induces STAT3 phos-
phorylation via IL-6 receptors and Janus family kinases (JAK), and is thereby involved in cell proliferation, angiogenesis and apoptosis (54,55). For hepatocellular carcinoma, celecoxib has already been demonstrated to inhibit IL-6/IL-6-receptor-
induced JAK2/STAT3 phosphorylation (56). In a study focusing on arthritis, celecoxib significantly reduced secretion of IL-6 and IL-8 in synovial fluid (57). Similarly, simvastatin was also shown to inhibit IL-6 and IL-8 production in rheumatoid arthritis (58). However, the effect of a combination of celecoxib and simvastatin on IL-6-secretion has not been investigated thus far, and in particular not for HNSCC.

Interleukin-8 (IL-8), one of the ELR\(^+\) CXC family of chemokines, is a potent pro-angiogenic factor and its expres-
sion is associated with angiogenesis, tumor progression and survival in patients with cancer (59,60). NSAIDs have been shown to have inhibitory effects on angiogenesis for pancreatic tumors in a mouse model, even by COX-independent mecha-
nisms (61), and have proven to inhibit other proangiogenetic factors such as matrixmetalloprotease (MMP)-2 and -9, as well as early growth response factor EGR-1 (62-64). Simvastatin could be demonstrated to inhibit the production of IL-6 and IL-8 as well as cell proliferation in patients with rheumatoid arthritis (58). To our knowledge, the present study is the first to evaluate the effect of a combination of celecoxib and simvastatin on IL-8 production, and therefore the angiogenesis of tumor cells, which may be of great value in the treatment of metastatic cancer considering the critical role of angiogenesis.

Still, there is much controversy about the concentrations of simvastatin and celecoxib used in in vitro experiments, regarding the expected in vivo dose necessary to achieve similar effects in humans (65). Most in vitro studies regarding cancer therapy have come to use concentrations of 40-60 µM of simvastatin (66). Although it is true that the in vitro concen-
trations generally used in oncologic studies are higher than the expected dose in vivo, there is also the problem of accumulation in the target organ, the liver, which at least partially makes up for the difference in concentrations (67). The same is valid for celecoxib, where the concentrations generally used in vitro are also slightly higher than the expected in vivo doses achiev-
able in humans (68). Therefore, to further reduce possible side effects when examining the co-medication in vivo, lower concentrations should also be tested for possible further syner-
gistic effects in the future.

Since especially simvastatin intervenes in the synthesis of Cholesterol, a key in cellular integrity, there could be possible damage to regular human cells as well. Gauthaman et al., on the other hand, already showed that simvastatin decreased viability
and proliferation of cancer cells and cancer stem cells, but had no effect on normal human stem cells (69). This, coupled with the years of clinical practice and experience with possible side effects of both substances, may still make it worthwhile to further examine their possible use as anticancer drugs.

In conclusion, it could be demonstrated that a combination of celecoxib and simvastatin has significant synergistic effects on reducing tumor cell proliferation and viability in HNSCC cells in vitro. These antitumor effects are based on apoptosis and cell cycle arrest in G0/G1-phase. Furthermore, a reduction in the secretion of IL-6 and IL-8 could be shown, indicating additional ways this synergism works to inhibit tumor growth, such as via antiangiogenesis. At present, medical tumor therapy for HNSCC is still limited. However, since both substances have a good risk-benefit ratio based on their long-term clinical use as lipid-lowering and anti-inflammatory drugs, at least in concentrations used in the present study, their combined use for cancer therapy clearly warrants further investigation. Future studies will need to elucidate the intracellular mechanisms behind these effects. Especially analysis of mitogenic and other signaling pathways are relevant targets for further investigations.

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References


