The effect of the histone deacetylase inhibitor resminostat on head and neck squamous cell carcinoma cell lines

Doctoral thesis at the Medical University of Vienna for obtaining the academic degree

Doctor of Medical Science

Submitted by

Dr. med. univ. Elisabeth Foki

Supervisor:
Ass. Prof. Priv. Doz. Dr. Gregor Heiduschka
Department of Otorhinolaryngology
Medical University of Vienna
Department of Otorhinolaryngology
Waehringer Guertel 18-20
1090 Vienna

Vienna, 02/02/2017
I. Declaration

I hereby declare, that I have substantially contributed to the study presented in this thesis:

I am responsible for the study design and for the performance as well as for the analysis of all experiments.

Dr. Lorenz Kadletz contributed significantly to immunohistochemistry.

Dr. Isabella Stanisz supported the conduction of the clonogenic assays and FACS analysis.

Dr. Ulana Kotowski supervised the study during the absence of Prof. Dietmar Thurnher and Prof. Gregor Heiduschka.

Prof. Rudolf Seemann performed the statistical analysis of the clonogenic assays.

Dr. Rainer Schmid assisted the irradiation of the cells.

Prof. Dietmar Thurnher supervised the study and is responsible for the study design.

Prof. Gregor Heiduschka is responsible for the study design. He supervised the performance and statistical analysis of all experiments. Further, he supervised the completion of the thesis.

The work was done at the Departments for Otorhinolaryngology, Head- and Neck Surgery, Oral and Maxillofacial Surgery as well as Radiotherapy, Medical University of Vienna.

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IV. Abstract (English)

Introduction:
Head and Neck squamous cell carcinoma is the 6th common cancer worldwide. Treatment options include surgery, chemotherapy as well as radiotherapy. However, despite treatment advances, 5-year survival has not changed significantly during the last years. Histone deacetylase inhibitors are targeted therapies, which modify epigenetically DNA-conformations with the objective of preventing carcinogenesis and cancer progression by inhibition of histone deacetylation. With this study, we aimed to investigate the effect of histone deacetylase inhibitors on head and neck squamous cell carcinoma cell lines using the example of resminostat.

Methods:
The head and neck squamous cell carcinoma cell lines SCC25, CAL27 and FaDu as well as the human skin keratinocyte cell line HaCaT were treated with increasing doses of resminostat. After evidence of inhibition of cell proliferation, apoptosis was measured by flow cytometry. Synergistic effect of combined treatment with cisplatin or irradiation was investigated. Influence of treatment on Mcl-1, survivin and p-AKT expression was measured by Western Blot.

Results:
Resminostat showed an anti-proliferative effect in all cell lines. Synergism with cisplatin as well as with irradiation was evident. FACS analysis showed significant induction of cell death. Most interestingly, resminostat provoked a time- and dose-dependent down-regulation of survivin in all three head and neck squamous cell carcinoma cell lines.

Conclusion:
Resminostat is a new and promising agent in treatment of head and neck squamous cell carcinoma. Further, clinical testing will be required to prove this effect in vivo.
V. Zusammenfassung (Deutsch)


Material und Methodik:

Resultate:
Es zeigte sich ein antiproliferativer und synergistischer Effekt von Resminostat gemeinsam mit Cisplatin oder Strahlentherapie auf die drei Plattenepithelkarzinomzelllinien. Apoptose wurde nachgewiesen. Außerdem bewirkt Resminostat eine zeit- sowie dosisabhängige Hemmung der Survivin-Expression bewirkt.

Zusammenfassung:
Reminostat ist ein vielversprechender Wirkstoff im Kampf gegen den Krebs. Weitere klinische Testung sind jedoch notwendig, um den Effekt in vivo zu beweisen.
VI. Abbreviations

DNMT = DNA methyl transferase  
HDAC = Histone deacetylase  
DNMTI = DNA methyl transferase inhibitor  
HDACI = Histone deacetylase inhibitor  
HNSCC = Head and neck squamous cell carcinoma  
HPV = Human papilloma virus
VII. Publication arising from this thesis:

First of all I want to express my gratitude to the supervisor of this thesis, Ass. Prof. Priv. Doz. Dr. Gregor Heiduschka, for his guidance and support throughout the performance of this thesis.

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Further, I would like to acknowledge my laboratory and my co-workers, Dr. Ulana Kotowski, Dr. Lorenz Kadletz, Dr. Isabella Stanisz, Helga Martinek, Prof. Rudolf Seemann, Dr. Rainer Schmid and Prof. Dietmar Thurnher for their support and their assistance during this work.

Finally, I want to thank my husband, my family and my friends for supporting and motivating me all along the way.
IX. Aims of this thesis

To investigate the effect of resminostat on cell proliferation of head and neck squamous cell carcinoma cell lines in order to evaluate new targeted therapies for this aggressive carcinoma.

To evaluate its effect on current treatment options such as chemotherapy with the example of cisplatin, which is the standard drug, and irradiation.

To investigate the effect on protein expression of resminostat in immortalized head and neck squamous cell carcinoma cell lines.
1. Introduction

1.1. General introduction in head and neck squamous cell carcinoma

Squamous cell carcinoma of the head and neck (HNSCC) is the 6th common cancer accounting for more than 600,000 cases worldwide in 2012. The term head and neck cancer specifies any cancer of the aerodigestive tract and includes mucosal cancer, nasopharyngeal cancer, sinu-nasal carcinoma, salivary gland cancer, non-melanoma cutaneous cancer as well as squamous cell carcinoma of unknown primary. Cancer originating from the squamous epithelium of the head and neck accounts for 90% of all cases of head and neck cancer.

Main risk factors are tobacco and alcohol consumption, betel nut chewing and HPV infection. Cancer of the lip is associated with UV-exposure. Tobacco attributes to 80% squamous cell carcinoma of the head and neck cases and its effect is multiplicative with heavy alcohol consumption. Smokers are 10-times more at risk for developing HNSCC than never-smokers. Significance of head and neck carcinoma has been rising with the emergence of human papilloma virus (HPV) infection as cause for oropharyngeal cancer. It mainly affects adults between 40 and 55 and is associated with promiscuous sexual behavior. Approximately 150 subtypes of HPV exist, which can be divided in “high-risk” and “low-risk” HPV tribes. Among the high risk types HPV 16 and 18 are the most carcinogenic.

Treatment choices depend on stage and localization of the primary carcinoma as well as on presence of metastasis. Treatment options are surgery, chemotherapy with cisplatin, and or docetaxel and 5-fluorouracil as well as immunotherapy with cetuximab, which is a monoclonal antibody. Additionally or alternatively, HNSCC can be treated with irradiation. However, some patients seem to be resistant to certain chemotherapies or radiation. Radioresistance seems to be related to mutations in EGFR-signalling, PI3K/mTOR-pathway and p53 signaling cascade.
1.2. Carcinogenesis

Healthy tissues regulate production and inhibition of growth-promoting signals by the cell cycle ensuring maintenance of regular tissue architecture. Cancer tissue consists of complex networks composed of multiple cell types which provide complex signaling cascades to sustain chronic proliferation.

In 2000, Hanahan and Weinberg proposed the six hallmarks of cancer, which determine carcinogenesis: evasion of programmed cell death (apoptosis), sustained angiogenesis, invasion of healthy tissue and metastasis, limitless reproductive potential, self-sufficiency in growth signals and insensitivity to anti-growth signals.

In 2006 progressive research revealed two new hallmarks determining carcinogenesis as well: the ability to modify or alter cellular metabolic processes and the capability of immuno-evasion facilitated repair mechanisms of innate immune cells. Those new hallmarks are supported by genomic instability and mutations.

Figure 1: Characteristics of carcinogenesis.
1.3. Epigenetics

Carcinogenesis can either occur as a result of altered gene expression programs or because of histone modifications having an influence on genome architecture and/or chromosome segregation. However, in contrast to mutations in the DNA, histone modifications are reversible and therefore offer a big opportunity for drug treatment of cancer 14.

Cell types hold tissue-specific epigenomes, that determine existence of multicellular organisms and differentiation of various tissues 15. The term “epigenetics” is defined as heritable cellular information other than DNA sequence and implies DNA methylation, histone modification and genomic imprinting 16. The “hallmarks of cancer” are promoted by epigenetically dysregulated genes. Certain mutant genotypes enable the survival and outgrowth of selected sub-clones of cells. Increased sensitivity to mutagenic agents is achieved by breakdown in one or several components of the genomic defense 12.

The macromolecular complex, which contains the eukaryotic genome, is Chromatin. The fundamental element of Chromatin is the nucleosome. This complex contains 147 base pairs of DNA which are wrapped around a histone octamer consisting of two of each histones H2A, H2B, H3 and H4 and non-histone proteins 17,18. Repeats of nucleosomes are linked by a particular “linker DNA”. Chromatin can be subdivided in two functional conformation states: heterochromatin, which is condensed and thus inactive, and euchromatin, which is de-condensed and transcriptionally active 17,18. Epigenomic modifications can occur through histone methylation and histone modification 19. Modifications to DNA and transcription of genes are carried out by histone-modifying enzymes which alter chromatin structure by non-covalent interactions within the nucleosome 17,20.
1.3.1. DNA methylation

DNA methylation is a dynamic process that either alters or erases chromatin modification. Changes occur on 5-carbon cytosine residues of CpG dinucleotides within the DNA and mainly alters structure of telomeres, centromeres, repeat sequences and inactive X-chromosomes. Abnormal methylation of normally unmethylated gene promoter CpG islands and transcriptional silencing implicates loss of gene function and occurs in 5-10% of CpG islands. Baylin et al. showed that these hyper-methylated CpG islands affect expression of protein coding genes as well as expression of noncoding RNAs, both of it playing a role in carcinogenesis. It also allows binding of proteins which again recruit histone-modifying enzymes. DNA methylation and histone modification is associated with cancer development and progression. In carcinogenesis, the amount of hypo-methylated DNA correlates with disease progression. Hypo-methylating agents as decitabine have already been approved by the FDA and show success in various human cancers.

1.3.2. Histone modification

In addition to DNA modification, post-translational histone modifications induce a „histone code“ which can also be modified by a crosstalk of various histone-modifying enzymes among each other. Histone modification such as acetylation, phosphorylation, sumoylation, ubiquitination, methylation and less commonly by citrullination and ADP-ribosylation are very sensitive in the regulation of all DNA-based processes and happens at the N-terminal histone tail in chromatin. They appear to be very organ and tissue-specific. Histone methylation and acetylation seem to be most important in development of human cancers and are of a clonal nature indicating disruption in early generations of tumor development.

Histones are methylated on the side chains of lysine, arginine and histidine residues by Histone methyltransferases. Histone methylation is a dynamic process and the
reverse reaction is demethylation of histones. Methylation at H3K4, H3K36 and H3K79 causes transcriptional activation whereas H3K9, H3K27 and H3K20 methylation is associated with repression of transcription 28. Histonemethylation is cross-linked with other histone modifying procedures, g.e. deacetylation of a given lysine residue may allow its further modification by histone methyltransferases 29.

1.3.3. Histone acetylation and deacetylation

Histone modification by acetylation can alter cell fate by mRNA splicing, mRNA transportation, mRNA integrity, translation, protein activity, protein localization, protein stability and protein-interaction 24. Histone acetylation happens post-translationally at the e-amino groups of lysine residues present within the N-terminal extensions of the core histones and is a complex mechanism, executed by histone acetyl transferases (HAT) which use acetyl Co- as a co-factor and Histone deacetylases (HDAC) 30. Histone acetylases modify histones by acetylating lysine residues of positively charged histones. This neutralization impairs interaction between histones and negatively charged DNA. As a consequence these two groups of enzymes provide a steady state that requires a lot of energy but also allows quick up- or down-regulation of gene transcription 31. This results in a high turnover of alternating acetylation and deacetylation of histones acetates. Histone acetyl transferases provoke chromatin decondensation whereas deacetylation causes chromatin compaction by removing the acetyl group of histones and is often associated with gene repression 32.

Histone hyperacetylation provides accessibility of DNA to transcription factors whereas deacetylation causes suppression of gene transcription 20. However, Histone deacetylation can also inhibit binding of transcription factors and can moreover influence cellular regulatory processes from signaling to protein degradation. 24. They have emerging importance because of their non-histone substrates as pRB, p53, STAT3 and NFκB 24.
1.4. Groups of Histone deacetylases

There are 30 types of HAT’s known so far, all located either in the cytosol or in the nucleus.

18 existing HDACs are known, which can be subdivided in four subgroups: class I consisting of HDAC 1, 2, 3, and 8 (nuclear location); class II containing HDAC 4, 5, 6, 7, 9, and 10 (localized in nucleus and cytoplasm); class III contains sirtuins (SIRT1-7); and class IV comprises HDAC 11, which has similarities to HDACs of Class I and II.

Class I, II, and IV HDACs are similar in structure and need a zinc ion as co-factor. In contrast, sirtuins are unique in their sequence or structure and require nicotinamide adenine dinucleotide (NAD+) for their catalytic activity.

Class I HDACs are expressed in all tissues and HDAC 1 and 2 are involved in regulation of cell cycle, apoptosis and proliferation and thus are deregulated in many cancers. Overexpression of HDAC class 1 has been observed in many cancers such as lung cancer and gastric cancer and has moreover been associated with poor prognosis, advanced stage and strong proliferative activity. However, also mutations of class I HDACs enabling carcinogenesis have been observed. In epithelial cancers microsatellite instability of HDAC 2 and resulting loss of its expression is linked to resistance to HDAC inhibitor treatment. In oral squamous cell carcinoma higher HDAC 2 expression was correlated with advanced stage, larger tumor size and lymph node metastasis resulting in reduced overall survival. Apparently, dysregulation as well as expression of HDACs can result in diverse cellular effects being expressed in healthy as well as in cancerous tissue.

In normal tissue HDAC class I enzymes are expressed in fibroblasts and myofibroblasts, epithelial cells, inflammatory cells and macrophages.

Class II:
Class II HDACs can be subdivided in class IIa and class IIb.

HDACIIa enzymes are responsible for cell differentiation and development and exist in particular tissues such as skeletal, cardiac and smooth muscle, bone, the immune
system, the vascular system an the brain where the act as transcriptional repressors. Their regulatory N-terminal domain subjected for phosphorylation, that interacts with tissue-specific transcription factors and co-repressors 35,42. They also obtain a conserved deacetylase domain with unknown function. This data indicates that depending on the cellular context, class IIa HDACs either obtain oncogenic or tumor suppressive functions.

Class IIb contains HDAC 6 and 10 and contain two specific deacetylase domains. They exist in the nucleus and in the cytoplasm. However, their particular function remains unknown 35. HDAC 6 is significantly overexpressed in oral squamous cell carcinoma and its expression correlates with advanced stage 43.

Class III HDACs:
Sirtuins or class III HDAC play a dual role in cancer and are found either in the cytosol, the nucleus or in the mitochondria 44. They promote posttranslational acetylation (SIRT 1, 2, 3 and 5) or ADP ribosylation (SIRT 4 and 6). SIRTS mediate chronically stress-induced cell senescence as well as promote cell survival by acetylation 44.

Class IV HDAC:
HDAC class IV consists of HDAC 11 solely. Feng et al. showed that inhibition of HDAC 11 by siRNA or trichostatin A increased the acetylation of transcription factors, suggesting an inhibitory mechanism via the ARHI tumor suppressor gene in breast cancer cells 45. Moreover, HDAC 11 overexpression is associated with the occurrence of philadelphia-negative chronic myeloproliferative malignancies 46.
1.5. Histone deacetylase inhibitors

Targeting epigenetic constellations in order to prevent carcinogenesis or cancer progression provides a reason for the use of targeted therapies. Currently, there are two classes of drugs for therapeutic use within this field available. These drugs inhibit enzymatic activity of either DNMTs or HDACs in order to provoke transcriptional silencing. HDAC inhibitors can be classified depending on their chemical structure into hydroxamates, cyclic peptides, benamides and fatty acids or according to their specificity for enzymes. Especially in hematological malignancies various HDACIs and DNMT inhibitors have been approved for clinical use. HDACs influence the entire acetylome and are involved in management of the cell cycle and regulation of mitosis, the DNA damage response, cellular stress response, protein degradation, cytokine signaling, immunity and inflammation, angiogenesis and cell survival. Because of this plethora of different functions in the cell, HDAC inhibition can cause a broad spectrum of cellular changes, including apoptosis in cancer cells.

Since HDAC inhibitors act as enzyme inducers, the specific pharmacology of them is particularly complex as they can influence their own kinetics as well as the kinetics of simultaneously administered drugs. HDAC inhibition results in acetylation of other proteins as well, such as p53, STAT signaling and Hsp90 that influence drug resistance or induce signaling of unrelated pathways. The effect of HDAC inhibitors can be measured by its enzymatic activity, but as well, acetylation of H3 and H4, can be used as biomarkers. An increase of H3 acetylation in peripheral blood cells at effective doses has been correlated with several drugs, even if intratumoral H3 acetylation status did not necessarily correlate with it.

Various histone deacetylase inhibitors have been established in daily clinical routine. Valproic acid is a standard drug in treatment of epilepsy. The first histone deacetylase inhibitor suberoylanilide hydroxamic acid (vorinostat, SAHA) has been approved by the FDA in 2006 and has shown good results in refractory cutaneous T-cell lymphoma.
In HNSCC, romidepsin achieved success in tumor treatment in phase II trials although tolerability is still improvable \(^5\). However, romidepsin has been approved by the FDA for cutaneous T-cell lymphoma \(^4\).

The inhibitory capacity of various HDAC inhibitors depends on the Zn\(^{++}\)dependency of HDAC enzymes \(^5\). A classic example of this is trichostatin A (TSA), which was one of the first investigated HDAC inhibitors.

1.5.1 Resminostat

A new promising agent in anti-cancer therapy is resminostat, a HDAC inhibitor which interacts with AKT-signaling pathway \(^5\) which is often dysregulated in HNSCC. This pathway predicts bad clinical outcome in HNSCC \(^5\). Recent studies have shown an effect of resminostat in various tumors \(^5\), but so far it has not been tested in head and neck squamous cell carcinoma cell lines. In this study we aim to investigate the effect of resminostat, a new HDAC-Inhibitor on head and neck squamous cell carcinoma cell lines.

![Figure II: Chemical structure of resminostat.](image-url)
1.6. PI3K-signaling

EGFR/PI3K/AKT/mTOR–pathway is a frequently dysregulated pathway in all cancers. 20-50% of HNSCC harbor mutations in the PI3KCA–gene with a predominancy of in advanced stage and HPV positive carcinomas. Targeting of EGFR/PI3K/AKT/mTOR–signaling happens downstream of ERBB–tyrosine kinase receptor family, whose most famous member is EGFR. Thus, activation of this pathway can occur by upstream targeting of up mentioned tyrosine kinases but also by dysregulation of each member the pathway itself.

Active phosphoinositide–3 kinase (PI3K) creates phosphatidylinositol-3,4,5-trisphosphate by phosphorylation of phosphatidylinositol-4,5-bisphosphate. The most central tumor supressor in this cascade is PTEN, which influences negatively PI3K acvitvation. Generation of phosphatidylinositol-4,5-bisphosphate results in phosphorylation of AKT and as a consequence leading to mTOR activation, and in turn, resulting in activation of multiple targets causing among others cell proliferation, cell growth and apoptosis.
1.7. Apoptosis

Apoptosis is a form of programmed cell death and is operated by caspases \(^{65}\). Apoptosis is regulated by an intrinsic and an extrinsic pathway, the latter being important for immune selection and inflammation and is mediated by TNF-\(\alpha\) and FAS (CD95) \(^{66}\). The first is mediated release of cytochrome c \(^{67}\) and SMAC/DIABLO \(^{68}\) from the mitochondria in the cytoplasm in order to provoke caspase-activation. Two families of important apoptotic regulating proteins exist: the family of BAX/BCL-2 and the family of anti-apoptotic proteins \(^{66}\).

In this study, we investigated two anti-apoptotic proteins and their reaction on treatment with resminostat.

1.7.1. Survivin

Survivin (BIRC5) is a cell-cycle protein belonging to the group of anti-apoptotic proteins, which, as the name suggests, inhibit apoptosis by binding capsases \(^{69}\). Together with XIAP, cIAP -1 and -2, survivin is characterized by a N-terminal baculovirus IAP repeat \(^{66}\). Survivin is expressed in HNSCC and its down-regulation influences radioresponse positively \(^{70}\). Inhibition of survivin leads to reduced phosphorylation of p-AKT and p-mTOR and suppresses tumor growth in HNSCC \(^{71}\).

1.7.2. Mcl-1

Mcl-1 (Myeloid cell leukemia sequence – 1) is a member of the anti-apoptotic BAX/BCL-2 family and is being reported to be expressed in HNSCC \(^{72}\). Members of the BAX/BCL-2 family counteract the pro-apoptotic BAX and BAK proteins, being part of the intrinsic apoptotic pathway \(^{73}\).
II. Materials and Methods

2.1. Cells and reagents

The HNSCC cell lines SCC25 and FaDu were purchased from the American Type Culture Collection (Manassas, VA, USA). CAL27 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The immortalized human keratinocyte cell line from human skin was obtained from Addex Bio (San Diego, CA, USA). Cell lines were grown in RPMI medium (Cambrex, Walkersville, MD, USA) supplemented with 10% fetal calf serum (PAA Laboratories, Linz, Austria) and 1% Penicillin/Streptomycin (Gibco BRL, Gaithersburg, MD, USA) and incubated at 37°C in an atmosphere of 5% CO2.

2.2. Drugs

Resminostat (RAS2410) was obtained by Selleck Chemicals (Houston, TX, USA), diluted in dimethylsulfoxide (DMSO) and was stored in 100μmol stock at −20°C. Cisplatin was stored at −4°C as ready-to-use infusion.

2.3. Cytotoxicity assay

A cell counting kit–8 cell proliferation (CCK-8) assay (Dojindo Molecular Technologies, Gaithersburg, MD, USA) was used to investigate the antiproliferative effect of resminostat on HNSCC cells. Cells were seeded into 96-well plates at a density of 3x10^3/ well. After 24 hours of growth, the cells were treated with resminostat and cisplatin, either alone or in combination and incubated for 72 hours. Untreated cells maintained in RPMI and equal concentrations of DMSO served as control. After 72 hours cell proliferation was measured by CCK-8 according to
manufacturer's protocol. In particular, 10 µl of a tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] solution was added to each well. WST-8 is reduced by dehydrogenases in cells resulting in an orange colored product dissolved in RPMI. The amount of the dye correlates positively with the number of living cells. After 1 hour of incubation, the absorbance of living cells was measured by a 450nm wavelength microplate reader. Experiments were carried out three times. IC50 of resminostat and cisplatin alone was calculated using Prism 5.0 (Graphpad software Inc., San Diego, CA, USA).

2.4. Irradiation

After treatment with increasing doses of resminostat as described above, cells were irradiated with 2, 4, 6 or 8 Gray (Gy) as single treatment using a conventional 150kV x-ray radiation source. Thermoluminescence dosimetry was performed in order to measure the radiation dose for the following experiments. After 72 h, growth inhibition was assessed by CCK-8 assay and CI values were calculated as described previously.

2.5. Colony forming assay

To assess long-term effect of resminostat on irradiated cells, clonogenic assays were conducted according to the protocol of Franken et al. In brief, 3×102 -12×102 cells were seeded into 6-well plates well plate. After 24 hours cells were treated with 1.25µM or 2.5µM resminostat and directly after irradiated with 2, 4, 6, or 8Gy using a conventional radiation source with 150kV x-ray machine as previously described. Untreated and unirradiated cells served as a control group. After 72 h, drug-containing medium was replaced by drug-free RPMI medium. After 10 days of incubation, cells were washed twice with PBS, and after fixation with methanol,
stained with methylene blue. Colonies with more than 50 cells were counted as surviving cells. The surviving fraction was calculated according to the model published by Franken et al. 75.

2.6. Flow cytometry analysis

1x10^5 cells were seeded in 6-well plates. After 24 hours, cells were treated with 2.5µM or 5µM resminostat and allowed to grow for 48 hours. Then, apoptosis was measured using AnnexinV-Apoptosis Detection System (Bender Medstems, Vienna, Austria). In brief, supernatant was collected and living cells were detached by the addition of accutase (SigmaAldrich, St. Louis, MO, USA). After incubation for 20 minutes, PBS was added to the cells in order to wash them and to inactivate the enzymatic activity of the accutase. After centrifugation, Annexin and PI was added and analysis was performed according to the protocol. Apoptosis was defined as Ann+/PI-. As differentiation between late apoptosis and necrosis is not valid in this essay Ann+/PI+ as well as Ann-/PI+ was defined as necrosis.

2.7. Western Blot

5x10^5 cells were seeded in 10 cm culture dishes and allowed to grow for 24 hours. Then, cells were treated with either 1.25µM, 2.5µM resminostat, cisplatin or either combination of cisplatin. In case of assessment of the effect of irradiation on protein expression, cells were irradiated directly after treatment with 4 and 8 Gy. After 24 and 48 hours cells were washed with PBS, frozen with liquid nitrogen and treated with lysis buffer as described previously. The lysates were centrifuged at 14,000 rpm at 4°C for 20 minutes. Protein concentrations of the supernatants were assessed using Micro BCA protein counting kit from Pierce (Rockford, IL, USA). 20µg of protein was separated by SDS-PAGE (10%) and electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). After blocking with 5% BSA in PBS-Tween overnight, membranes were incubated with the appropriate diluted primary antibody
anti-Mcl-1 (Neomarkers, CA, USA), anti-p-AKT (Cell signalling technologies, Danvers, MA, USA) and anti-survivin (Abcam, Cambridge, UK). Antigen binding was visualized with the Immun-Star Western C Kit (Bio-Rad Laboratories, CA, USA) and detected by ChemiDoc-It Imaging System (UVP, CA, USA).

2.8. Immunohistochemistry

Formalin-fixed paraffin-embedded tissue was stained with a monoclonal antibody directed against HDAC 11 (Abcam, Cambridge, UK). Dewaxed and rehydrated tumor tissue samples were cut from each formalin-fixed paraffin-embedded block, whereas 1 section of each tissue sample was stained by hematoxylin-eosin to identify invasive tissue. Immunohistochemical staining of tumor samples was performed according to a protocol previously described \(^77\). Briefly, tissue sections were de-paraffinized with xylene and rehydrated with decreasing alcohol concentrations. After heat-induced epitope retrieval in a microwave oven (600W) using citrate buffer (10mmol/l, pH 6.0), slides were incubated at room temperature with the primary antibody directed against HDAC 11 in a dilution of 1:100 for one hour. Then, antibody binding was detected by means of the UltraVision LP detection system (Lab Vision Corporation, Fremont, CA) in accord with the manufacturers recommendations. Color development was achieved by 3,3’-diaminobenzidine and antibody binding locations stained brownish. Finally, slides were counterstained with Hematoxylin Gill III (Merck, Darmstadt, Germany). Samples were analyzed using an Olympus BH-2 microscope (Olympus, America, Center Valley, PA).

2.9. Statistical analysis

Statistical analysis for cell viability assays, clonogenic assays and flow cytometric experiments were performed by ANOVA using either Graph Pad 5.0 software by PRISM® (GraphPad Software Inc., San Diego, CA, USA) or SPSS® Version 21 software (IBM®). Dose-response curves were generated using Graph Pad 5.0. Interactions with cisplatin were calculated using CalcuSyn® (Version 2.0. Biosoft, Cambridge, UK) based on the Chou – Talalay equation \(^78\). The plating efficiency of
clonogenic assays were calculated by a protocol of Franken et al. \textsuperscript{75} using SPSS® Version 21 software (IBM®).

All experiments were repeated in triplets. P-values <0.05 were considered as statistically significant.
3. Results

3.1. Prologue

At the time of planning this diploma studies, the HDAC inhibitor resminostat turned out to be very potent in our cell lines. Thus, we focused on resminostat as representative example of HDAC inhibitors in HNSCC cell lines.
### 3.2. Publication

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The effect of the histone deacetylase inhibitor resminostat on head and neck squamous cell carcinoma cell lines
The effect of the histone deacetylase inhibitor resminostat on head and neck squamous cell carcinoma cell lines

Elisabeth Enzenhofer, MD¹, Lorenz Kadletz, MD¹, Isabella Stanisz, MD¹, Ulana Kotowski, MD¹, Rudolf Seemann, MD, DMD, MSc, MBA², Rainer Schmid, MD³, Dietmar Thurnher, MD¹, Gregor Heiduschka MD¹

¹Department of Otorhinolaryngology, Head and Neck Surgery, Medical University of Vienna, Vienna, Austria

²Department of Oral and Maxillofacial surgery, Medical University of Vienna, Vienna, Austria

³Department of Radiotherapy, Medical University of Vienna, Vienna, Austria

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* Corresponding author:

Gregor Heiduschka, MD, PD
Department of Otorhinolaryngology, Head and Neck Surgery
Medical University of Vienna
Waehringer Guertel 18-20
A-1090 Vienna
Tel.: +43 1 40400 2078
Fax: +43 1 40400 3355
Email: gregor.heiduschka@meduniwien.ac.at
Abstract

Background:

Carcinogenesis is determined by various epigenetic events such as histone deacetylation. The aim of this study is to investigate the effect of the new histone deacetylase inhibitor resminostat on head and neck squamous cell carcinoma (HNSCC) cell lines.

Methods:

The cytotoxicity of resminostat and cisplatin on HNSCC cell lines SCC25, CAL27 and FaDu was determined using CCK-8 cell proliferation assay and combination index analysis. Cells were irradiated with 2 - 8 Gray. Apoptosis was measured using flow cytometry and expression of Mcl-1, p-AKT and survivin was investigated.

Results:

Treatment with resminostat showed a decrease of cell proliferation of HNSCC cell lines. Also, a synergistic effect with cisplatin as well as with radiation treatment could be observed. Induction of cell death and dose-dependent down-regulation of survivin was evident in all cell lines.

Conclusion:

Resminostat is a promising treatment of HNSCC due to its anti-proliferative, chemosensitizing and radiosensitizing effects.
Introduction

Head and neck squamous cell carcinoma (HNSCC) is a heterogeneous group of tumors, which derive from the squamous epithelium of the oral cavity, pharynx and larynx. It is the 6th common cancer world wide representing 6% of all cancer cases. In advanced stage tumors, surgery can be very mutilating and quality of life in these patients is often impaired. Recent advances in the use of concomitant radiochemotherapy and the use of intensified radiotherapy have led to longer survival times in clinical trials. However, 5-year survival rates have not changed dramatically during the last 30 years\(^1\).

Cancer initiation and progression is determined by various genetic and epigenetic events, which alter gene expression. Epigenetics are defined as changes in gene expression that are not accompanied by changes in DNA sequence and are commonly based on DNA methylation and histone modification\(^2\). Histone modification happens post-translationally through acetylation and ubiquitination, phosphorylation, sumoylation and methylation\(^3\). Various enzymes catalyze these modifications. In particular, histone acetylation is a complex mechanism, executed by histone acetyl transferases (HAT). This process results in a high turnover of alternating acetylation and deacetylation of histones. Histone acetyl transferases provoke chromatin decondensation whereas deacetylation causes chromatin compaction and suppressing of gene transcription\(^4\). The counterpart of histone acetyl transferases are a group of enzymes called histone deacetylases (HDAC)\(^3\). Recently, histone deacetylase inhibitors were evaluated as prospective anticancer agents and as promoters of synergistic or additive effect with other antineoplastic treatments including radiation, chemotherapy as well as other reagents\(^5-7\).
A very well known HDAC inhibitor (HDACI), which has already been established in treatment of neurologic diseases, is valproic acid. In vitro studies showed an anti-proliferative effect on HNSCC cells as well as potentiation of the effect of cisplatin in cell lines as opposed to oral keratinocytes. Nevertheless, preclinical trials have shown that these HDACIs are only of limited effect in HNSCC.

In contrast, vorinostat, another HDACI has been approved by the food and drug administration for the treatment of cutaneous T-cell lymphoma in 2006. Previous studies have shown, that a combination of PI3K and AKT inhibitors caused an enhancement of cytotoxicity induced by HDACI in HNSCC cells in vitro.

The novel pan-HDAC inhibitor resminostat has been reported to be well tolerated in phase II studies of therapy-resistant advanced solid tumors. Brunetto et al. reported antitumor activity of resminostat by affecting the AKT signaling pathway, which plays a fundamental role in mediating cell survival and proliferation. AKT mediates cell survival by inactivation of pro-apoptotic factors such as FOXO and p53 and induction of NF-κB. It is known that AKT activation is associated with poor clinical outcome in HNSCC and thus, it is a potential target of resminostat in HNSCC.

Not only phosphorylated AKT (p-AKT) signaling, but also anti-apoptotic signaling in HNSCC is interesting for novel targeted therapies in HNSCC. Mcl-1, which is a major anti-apoptotic member of the Bcl-2 family and is frequently expressed in HNSCC, has been shown to be down-regulated after treatment with the HDAC-inhibitor entinostat and PI3K-blockage. Additionally, resminostat modulated various members of the Bcl-2 family in multiple myeloma cells.

Survivin belongs to a family of proteins known as inhibitor of apoptosis proteins that are crucial in the regulation of apoptosis and cell division. Survivin up-regulation in...
cancer cells promotes resistance to cancer therapies. Overexpression of survivin in cancer may overcome cell cycle checkpoints such as p53 activation to facilitate aberrant progression of transformed cells through mitosis and thus plays a key role in cancer progression. In gastric cancer, trichostatin A, an antifungal-compound and HDAC-inhibitor, provoked down-regulation of survivin and Mcl-1.

So far, no data on the effect of resminostat on cell survival of HNSCC with or without in combination with established anti-cancer treatment and its effect on protein expression is available. Thus, the aim of this study is to investigate the effect of the new histone deacetylase inhibitor resminostat on HNSCC cell lines.
Material and Methods

Cells and reagents

The HNSCC cell lines SCC25 and FaDu were obtained from the American Type Culture Collection (Manassas, VA, USA). CAL27 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The human immortalized keratinocyte cell line HaCaT was obtained from Addex Bio (San Diego, CA, USA). Cell lines were maintained in RPMI medium (Cambrex, Walkersville, MD, USA) supplemented with 10% fetal calf serum (PAA Laboratories, Linz, Austria) and 1% Penicillin/Streptomycin (Gibco BRL, Gaithersburg, MD, USA) and incubated at 37°C in an atmosphere of 5% CO2.

Drugs

Resminostat (RAS2410) was purchased by Selleck Chemicals (Houston, TX, USA), diluted in dimethylsulfoxide (DMSO) and was stored in 100 mol stock at ~20°C. Cisplatin was stored at ~4°C as ready-to use infusion.

Cytotoxicity assay

A CCK-8 cell proliferation assay (Dojindo Molecular Technologies, Gaithersburg, MD, USA) was used to investigate the anti-proliferative effect of resminostat on HNSCC cells. Cells were seeded into 96-well plates at a density of 3x10³ / well. After 24 hours of growth, the cells were treated with resminostat and cisplatin, either alone or in combination and incubated for 72 hours. Untreated cells maintained in RPMI and equal concentrations of DMSO served as control. After 72 hours cell proliferation was measured by CCK-8 according to manufacturer's protocol. Experiments were carried out in triplicates three times.
IC50 of resminostat and cisplatin alone were measured using Prism 5.0 (Graphpad software Inc., San Diego, CA, USA).

**Irradiation**

Cells were treated with increasing doses of resminostat as described above and consecutively irradiated with 2, 4, 6 or 8 Gray (Gy) as single treatment using a conventional 150kV x-ray radiation source. Thermoluminescence dosimetry was performed to measure the radiation dose for the following experiments. After 72 h, growth inhibition was determined by CCK-8 assay and CI values were calculated as described previously\(^2^0\).

**Colony forming assay**

To assess long-term effect of resminostat on irradiated cells, clonogenic assays were conducted according to the protocol of Franken et al\(^2^1\). In brief, 3\(\times\)10\(^2\) - 12\(\times\)10\(^2\) cells were seeded into 6-well plates. After 24 hours cells were treated with 1.25 M or 2.5 M resminostat and irradiated with 2, 4, 6, or 8Gy using a conventional radiation source with 150kV x-ray machine as previously described\(^2^0\). After 72 h, drug-containing medium was exchanged with drug-free RPMI medium. After 10 days, cells were washed twice with PBS, fixed with methanol and stained with methylene blue. Colonies with more than 50 cells were counted as the surviving fraction.

**Flow cytometry analysis**

1\(\times\)10\(^5\) cells were seeded in 6-well plates and allowed to attach for 24 hours. After 24 hours, cells were treated with 2.5 M or 5 M resminostat. After 48 hours apoptosis was measured using AnnexinV - Apoptosis Detection System (Bender Medsysystems, Vienna, Austria). Apoptosis was defined as Ann+/PI-. As differentiation between late
apoptosis and necrosis is not valid in this essay. Ann+/PI+ as well as Ann-/PI+ was defined as necrosis.

**Western blot**

$5 \times 10^5$ cells were seeded in 10cm culture dishes and allowed to grow for 24 hours. After 24 hours, cells were treated with subsequent concentrations. To measure the effect of resminostat alone, cells were treated with 1.25 M and 2.5 M resminostat. Subsequently, protein expression of survivin, p-AKT and Mcl-1 was determined after 24 hours. In order to measure the effect of resminostat in combination with cisplatin after 24 hours, cells were treated with 3.125 M resminostat, 2.5 M cisplatin as well as with a combination of these drugs. To investigate the effect of irradiation after 24 hours, cells were treated with 2.5 M resminostat and subsequently irradiated with 4 and 8Gy. Appropriate negative control was performed. After 24 and 48 hours cells were washed with PBS, frozen with liquid nitrogen and treated with lysis buffer as described previously. The lysates were centrifuged at 14,000 rpm at 4°C for 20 minutes. Protein concentrations were determined from the collected supernatants using Micro BCA protein counting kit from Pierce (Rockford, IL, USA). 20µg of protein was separated by SDS-PAGE (10%) and electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). After blocking with 5% BSA in PBS-Tween overnight, membranes were incubated with the appropriate diluted primary antibody anti-Mcl-1 (Neomarkers, CA, USA), anti-p-AKT (Cell signalling technologies, Danvers, MA, USA) and anti-survivin (Abcam, Cambridge, UK). Antigen binding was visualized with the Immun-Star Western C Kit (Bio-Rad Laboratories, CA, USA) and detected by ChemiDoc-It Imaging System (UVP, CA, USA).
Immunohistochemistry

Immunohistochemistry was performed after obtaining approval from the institutional research ethics board (the Ethics Committee of the Medical University of Vienna and Vienna General Hospital; REB 612/2009).

Formalin-fixed paraffin-embedded tissue was stained with a monoclonal antibody directed against HDAC 11. Briefly, dewaxed and rehydrated tumor specimens were cut from each formalin-fixed paraffin-embedded block, whereas one section was stained by hematoxylin - eosin to confirm and locate the tumor. Immunohistochemical staining of tumor samples was performed according to a protocol previously described. Briefly, tissue sections were de-paraffinized with xylene and rehydrated with decreasing alcohol concentrations. After heat-induced epitope retrieval in a microwave oven (600W) using citrate buffer (10mmol/l, pH 6.0), slides were incubated at room temperature with the primary antibody directed against HDAC 11 (Abcam, Cambridge, UK) in a dilution of 1:100.

Antibody binding was detected by means of the UltraVision LP detection system (Lab Vision Corporation, Fremont, CA) in accord with the manufacturers recommendations. Color development was achieved by 3,3'-diaminobenzidine whereas antibody binding locations stained brownish. Finally, slides were counterstained with Hematoxylin Gill III (Merck, Darmstadt, Germany). Samples were analyzed using an Olympus BH-2 microscope (Olympus, America, Center Valley, PA).

Statistical analysis

Statistical analysis for cell viability assays was analysed by 1-way ANOVA and flow cytometric experiments were performed by 2-way ANOVA using either Graph Pad 5.0 software by PRISM® (GraphPad Software Inc., San Diego, CA, USA) or SPSS®
Version 21 software (IBM®). Dose-response curves were generated using GraphPad 5.0. Interactions with cisplatin were calculated using CalcuSyn® (Version 2.0, Biosoft, Cambridge, UK) based on Chou – Talalay equation\textsuperscript{24}. All experiments were repeated in triplets. P-Values <0.05 were considered as statistically significant.
Results

Resminostat inhibits cell growth in HNSCC cell lines

Primarily, growth inhibition after treatment with resminostat was investigated. The three HNSCC cell lines SCC25, CAL27, FaDu and the immortalized keratinocyte cell line HaCaT cells were treated with increasing doses from 0 - 25 M. After 72 hours, a significant dose-dependent growth inhibition was seen in all tested HNSCC cell lines (p<0.001). IC50 values were calculated and ranged from 0.775 M to 1.572 M (IC50 for SCC25: 0.775 M, CAL27: 1.572 M, FaDu: 0.899 M). The IC50 value in HaCaT cells was 3.508 M. Dose-response curves for all cell lines are shown in figure 1.

Resminostat enhances cisplatin sensitivity

We further investigated the effect of concomitant treatment of resminostat and cisplatin on HNSCC cell lines. Cells were treated simultaneously with resminostat and cisplatin at a ratio of 1: 1.25 (figure 1). The effect was calculated with CalcuSyn® software and expressed as combination index (CI) versus fraction affected (Fa). CI· 1 corresponds to synergism, CI=1 indicates an additive effect and CI· 1 corresponds to antagonism. In all tested cell lines a synergistic effect was observed (figure 2). For IC50 values see table1.

Effect on cell survival after radiotherapy

To prove synergistic effect on irradiation cells were treated with resminostat and subsequently irradiated with 2, 4, 6 or 8Gy. First, cell viability assays were performed. Before irradiation, cells were treated with resminostat (0 – 25 M). After 72 hours, CCK-8 assay was performed. Surprisingly, we could not observe a short-time reduction of cell survival after irradiation (figure 3). The effect was analyzed
using CalcuSyn® software and expressed as combination index (CI) versus fraction affected (Fa). Since a constant dose ratio was not feasible, single CI values could only be calculated for particular combinations. CI<1 indicates a synergism, CI=1 indicates an additive effect and CI>1 corresponds to antagonism (table 2). However, to determine long time effect clonogenic assays were carried out. Treatment with 1.25 and 2.5 M resminostat has a synergistic effect with irradiation on HNSCC cell lines. An untreated control run with irradiated cells was performed (figure 4C). Treatment with resminostat significantly reduced clonogenic survival independently of treatment doses in all cell lines (p<0.001). Dose-response curves are shown in figure 4.

**Resminostat induces cell death**

As cell viability assays showed a dose-dependent reduction of cell proliferation, we evaluated induction of cell death. Previous data acquisition revealed best apoptosis detection after 48h, therefore treatment with 2.5 M and 5 M resminostat was performed. After 48 hours, experiments showed an increase in apoptotic and necrotic cell death in a dose-dependent manner. The best effect could be observed in SCC25 cells (apoptosis: p=0.03; total cell death: p=0.002). In CAL27 (apoptosis: p=0.14; total cell death: p<0.001) and FaDu (apoptosis: p=0.21; total cell death: p<0.001) a significant increase of cell death as compared to untreated control was observed (figure 5).

**Expression of HDAC 11 in HNSCC**

28 paraffin-embedded tissue samples of oropharyngeal squamous cell carcinoma were stained with antibodies directed against HDAC 11. Expression of HDAC 11 could not be verified in any of the tissue samples (data not shown).
Effect of resminostat on survivin, Mcl-1 and AKT signaling

Western blot analysis of survivin, Mcl-1 and p-AKT was performed in all three cell lines before and after 24 and 48 hours of treatment with resminostat. Most interestingly, we could show a time- and dose-dependent decrease of survivin expression after 24 hours and 48 hours of treatment (figure 6). After 24 hours, a reduction in survivin expression in CAL27 cells treated with 2.5 M resminostat was visible, whereas, after 48 hours a down-regulation in survivin was visible in all cell lines and all dosages. However in the SCC25 cell line a down-regulation was only visible in cells treated with 2.5 M.

In order to investigate the effect of the combination of cisplatin or radiotherapy with resminostat on survivin, Mcl-1 and p-AKT expression, Western blot was performed after 24 hours. In FaDu cells, neither treatment with cisplatin and combination nor with radiotherapy influenced survivin expression. In CAL27 cells, in contrast to treatment with cisplatin alone, treatment with resminostat and in combination with cisplatin or 8Gy induced a down-regulation of survivin. In SCC25 cells only the combination of cisplatin and resminostat induced a down-regulation of survivin (figure 6).

Mcl-1 and p-AKT expression was not affected by the treatment with resminostat (data not shown).
Discussion

In this study we demonstrate several anti-tumorigenic effects of the histone deacetylase inhibitor resminostat. Our experiments showed a synergistic effect of resminostat in combination with the standard chemotherapeutic agent cisplatin as well as a reduction of clonogenic survival after irradiation. We showed an increase in cell-death after treatment with resminostat. However, only in SCC25 cells, which derive from tongue oral squamous cell carcinoma, induction of apoptosis was significant. However, as late apoptosis and necrosis cannot be discriminated in our experiments, we sub-summarized both as total cell death, which was significant in all cell lines. A control run with a resminostat – treated human keratinocyte cell line showed that resminostat was much more effective in the HNSCC cell lines than in the keratinocyte cells, indicating a specificity for cancer cells.

In HNSCC various HDAC-enzymes have been described\(^{25}\). However HDAC 11 has not been described in HNSCC yet\(^{25}\). In this study, we investigated the expression of HDAC 11 in oropharyngeal squamous cell carcinoma tissue samples, but however, we could not show its expression in oropharyngeal squamous cell carcinoma.

Histone-deacetylase inhibitors are a promising group of various agents, which influence a variety of processes involved in histone modification. Potentially catastrophic and non-specific side effects as a consequence of global gene depression are still a matter of discussion. However, it is observable, that broad HDAC inhibition has been well tolerated in vivo\(^{16,26}\). So far, vorinostat, another PI3K/AKT interacting histone deacetylase inhibitor has been approved by the FDA for the treatment of cutaneous T-cell lymphoma in 2007.
Further, trichostatin A shows not only anti-fungal activity but also inhibits proliferation in cancer cells. It is used as reference substance within the group of HDAC inhibitors. Its HDAC inhibiting capacity lies within the nanomolar range. Few HDAC inhibitors have shown a similar potency.

Various HDACIs are dietary compounds such as sulforaphane, which has been published previously by our group as potent radiosensitizing agent in HNSCC.

Resminostat is an orally bioavailable pan-histone deacetylase inhibitor which has been shown to be efficient in various other tumor entities. It has been well tolerated in a phase I trial by patients with advanced solid tumors, with occurrence of only few side effects. To our knowledge, this is the first study to demonstrate the anti-proliferative as well as radiosensitizing effect of single treatment of resminostat on HNSCC cell lines. In this study, IC50 is ranging from 0.775 – 1.572 M which is in concordance with previously published results in other tumor entities and is comparable with the HDAC inhibiting capacity of trichostatin A. HDACIs have been developed but few show similar effectiveness.

Moreover, resminostat administered with subsequent irradiation reduced clonogenic survival, indicating a potent radiosensitizing effect. However, short time cytotoxicity did not reveal any synergistic effect of radiotherapy and resminostat. We performed a control run with untreated but irradiated cells, which revealed that our cells hardly respond to radiotherapy after 24 hours at all. Response to radiotherapy of HNSCC increases according to a linear-quadratic model with dose and time after treatment. This might be an explanation for the effect in this study, as radiosensitizing effect occurs only on the long term.

This theory is supported by the missing down-regulation - of major interest in SCC25
cells and FaDu cells - of survivin 24 hours after irradiation. Interestingly, only in the CAL27 cells, which is the most radiosensitive cell line in our short-time experiments, that had been irradiated with 8Gy, a change of expression was visible. As our short-term experiments were measured after 72 hours, whereas the long-term experiments were determined after 10 days, we conclude, resminostat exhibits its full radiosensitizing effect after more than 72 hours. Khan et al. reported about a radio- and chemosensitizing effect of survivin down-regulation in HNSCC, which supports our theory that survivin down-regulation by resminostat starts after 24 hours of treatment with resminostat.

Furthermore, combined administration with cisplatin reduced cell proliferation distinctively, reducing the IC50 to nanomolar ranges. Remarkably, resminostat was most potent in SCC25 cells, which are least sensitive to cisplatin in our experiments. Thus, in regard of lacking new anticancer agents in HNSCC resminostat could gain importance to overcome cisplatin resistance. Shen et al. reported on suberoylanilide hydroxamic acid to enhance cisplatin efficacy in oral squamous cell carcinoma, however lacking an anti-proliferative effect in single treatment. In contrast to suberoylanilide hydroxamic, one of the most potent HDAC inhibitors, resminostat showed strong anti-proliferative effects as single treatment.

In contrast to previous studies, resminostat did not interact with AKT-signaling, one of the key regulators in HNSCC. Our data suggests, that resminostat is either interfering with the AKT-pathway downstream of AKT or does not influence AKT-signaling at all. Moreover, expression of Mcl-1, an anti-apoptotic member of the Bcl-2 family, which is frequently expressed in HNSCC, was not affected by treatment with resminostat. However, we tested the interaction with survivin, a cell cycle protein which has anti-apoptotic properties, which has been considered as one of the most
tumor-specific proteins\textsuperscript{34,35}. Studies have shown that expression of survivin is associated with higher clinical tumor stage, presence of lymph node metastasis as well as with chemo- and radio-resistance in HNSCC\textsuperscript{36-40}. In our study, treatment with resminostat modified survivin expression in a time- and dose-dependent manner. In particular, after 24 hours of treatment, survivin down-regulation is only visible in CAL27 cells, whereas in after 48 hours a down-regulation of survivin was visible in all of the cell lines. The earlier down-regulation of survivin in CAL27 cells might contribute to the fact that CAL27 cells are more responsive to resminostat than the other cell lines.

After 24 hours, we could not show a survivin down-regulation in FaDu cells as well as in SCC25 cells treated with cisplatin and resminostat alone or in combination with radiotherapy. However, in SCC25 cells an effect on survivin expression of combined treatment is clearly visible. These effects might be explained by the fact that those experiments were carried out after 24 hours. Again, the strongest reduction of survivin expression after treatment with resminostat alone as well as in combination with cisplatin and radiotherapy was visible in CAL27 cells. This is corresponds to the data of the CI-Blots, where the strongest synergistic effect of survivin and resminostat could be measured in CAL27 cells. In all the cell lines, resminostat multiplies effectiveness of cisplatin many times over. This is consistent with a study of Kan et al who showed a relation between survivin expression and resistance to radio- and chemotherapy\textsuperscript{41}. Kumar et al. showed that treatment with the survivin inhibitor YM155 reverses resistance to cisplatin treatment\textsuperscript{42}. Further, survivin down-regulation has been linked to successful cancer treatment in HNSCC\textsuperscript{43}. Thus, treatment with resminostat might effect cell survival by decreasing expression of survivin in HNSCC.
To conclude, the results of this study show that resminostat is an effective anti-proliferative, chemo- as well as radiosensitizing substance in HNSCC cells. Enhancement of the treatment effect of cisplatin as well as of irradiation indicates that resminostat could be a potent anti-cancer drug administered especially concomitantly to radiotherapy or chemotherapy in future. However, further studies are required to confirm this effect in vivo.
Figure legends

Figure 1: Dose-response curves after treatment with resminostat alone or in combination with cisplatin. HNSCC cell lines SCC25, CAL27 and FaDu cells (A-C) were treated with increasing drug dosages of resminostat and cisplatin in a ratio 1.25:1. The human keratinocyte cell line HaCaT (D) was treated with increasing doses of resminostat (0-25 M). Error bars indicate standard error of the mean.

Figure 2: Combination index (CI) plots for treatment with resminostat in combination with cisplatin. CI values \( \leq 1 \) represent an additive effect, CI >1 indicates an antagonistic effect and CI values <1 represent a synergistic effect.

Figure 3: Dose-response curves after treatment with resminostat and subsequent irradiation with 0, 2, 4, 6, and 8Gy (A-C). Figure 4D shows a control run with native cells treated with 0, 2, 4, 6 and 8Gy radiotherapy. Error bars indicate standard error of the mean.

Figure 4: Long-term radiosensitizing effect was measured by assessment of clonogenic survival. Cells (A-C) were treated with 1.25 M and 2.5 M resminostat and subsequently irradiated with 0, 2, 4, 6 and 8Gy. Clones were counted after 10 days of incubation.

Figure 5: Assessment of apoptosis by flow cytometry. A) SCC25, B) CAL27 and C) FaDu cells were treated with 2.5 M and 5 M resminostat, respectively. Significant
induction of cell death is indicated by (*). Total cell death was significant in all three cell lines.

Figure 6:

Western blot analysis of survivin (s) and tubulin (t) expression. In each of the cell lines (SCC25, CAL27 and FaDu), 4 different treatment conditions (A and B) or 3 different treatment conditions (C and D) were applied. The symbols at the end of each section describe the respective treatment condition in each cell line.

(A) Western blot of survivin expression after treatment with 2.5 M resminostat (+), in combination with 4Gy (+#) and 8Gy (+##). Untreated cells serve as control (-).

(B) Western blot of survivin expression after treatment with 3.125 M resminostat (+), with 2.5 M cisplatin (*) and both drugs together (+*). Untreated cells serve as control (-).

(C) Western blot of survivin expression after 24 hours of treatment with 1.25 M resminostat (+) and 2.5 M resminostat (++). Untreated cells serve as control (-).

(D) Western blot of survivin expression after 48 hours of treatment with 1.25 M resminostat (+) and 2.5 M resminostat (++). Untreated cells serve as control (-).

Table legends

Table 1: IC50 for resminostat, cisplatin and combination of both drugs, respectively.

Table 2: CI values for treatment with resminostat and radiotherapy in combination after 72 hours. CI\cdot 1 corresponds to synergism, CI=1 indicates an additive effect and CI\cdot 1 corresponds to antagonism. N/A=data not available.
References


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Dose-response curves after treatment with resminostat alone or in combination with cisplatin. HNSCC cell lines SCC25, CAL27 and FaDu cells (A-C) were treated with increasing drug dosages of resminostat and cisplatin in a ratio 1.25:1. The human keratinocyte cell line HaCaT (D) was treated with increasing doses of resminostat (0-25µM). Error bars indicate standard error of the mean.

169x100mm (300 x 300 DPI)
Combination index (CI) plots for treatment with resminostat in combination with cisplatin. CI values =1 represent an additive effect, CI >1 indicates an antagonistic effect and CI values <1 represent a synergistic effect.
Dose-response curves after treatment with resminostat and subsequent irradiation with 0, 2, 4, 6, and 8Gy (A-C). Figure 4D shows a control run with native cells treated with 0, 2, 4, 6 and 8Gy radiotherapy. Error bars indicate standard error of the mean.

149x88mm (300 x 300 DPI)
Long-term radiosensitizing effect was measured by assessment of clonogenic survival. Cells (A-C) were treated with 1.25µM and 2.5µM resminostat and subsequently irradiated with 0, 2, 4, 6 and 8Gy. Clones were counted after 10 days of incubation.
Assessment of apoptosis by flow cytometry. A) SCC25, B) CAL27 and C) FaDu cells were treated with 2.5µM and 5µM resminostat, respectively. Significant induction of cell death is indicated by (*). Total cell death was significant in all three cell lines.

103x244mm (300 x 300 DPI)
Western blot analysis of survivin (s) and tubulin (t) expression. In each of the cell lines (SCC25, CAL27 and FaDu), 4 different treatment conditions (A and B) or 3 different treatment conditions (C and D) were applied. The symbols at the end of each section describe the respective treatment condition in each cell line.

(A) Western blot of survivin expression after treatment with 2.5µM resminostat (+), in combination with 4Gy (+#) and 8Gy (+##). Untreated cells serve as control (-).

(B) Western blot of survivin expression after treatment with 3.125µM resminostat (+), with 2.5µM cisplatin (*) and both drugs together (+*). Untreated cells serve as control (-).

(C) Western blot of survivin expression after 24 hours of treatment with 1.25µM resminostat (+) and 2.5µM resminostat (++). Untreated cells serve as control (-).

(D) Western blot of survivin expression after 48 hours of treatment with 1.25µM resminostat (+) and 2.5µM resminostat (++). Untreated cells serve as control (-).
Table 1: IC50 values of treatment with resminostat, cisplatin or in combination.

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<th>FaDu</th>
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Table 2: CI values of treatment with resminostat, radiotherapy or in combination. CI=1 indicates an additive effect, CI<1 a synergistic effect and CI>1 antagonism. N/A=data not available.

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4. Discussion

4.1. General Discussion

With the present study we aimed to investigate a new option for the treatment of head and neck squamous cell carcinoma. Squamous cell carcinoma of the head and neck is the 6th common cancer worldwide and 5-year survival has not improved during the last years. Surgery, radiotherapy and chemotherapy are the main treatment options. Despite continuous advances in surgery and preservation of organ function, substantial functional and cosmetic restrictions hint at the advantages of conservative treatment options such as chemo- or radiotherapy in order to maintain quality of life. Radiotherapy of HNSCC, causing severe damage of tumor DNA, is the most effective non-surgical treatment option. Success of radiotherapy is dependent on primary site, smoking and factors such as HPV status. HPV positive patients are more sensitive to radiotherapy and thus primary radiochemotherapy has been recommended for oropharyngeal squamous cell carcinoma. A recent meta-analysis showed that combination of radiotherapy with chemotherapy is more effective in treatment of HNSCC than radiotherapy alone. However, non-surgical treatment also harbors severe side effects and even more, some patients do not respond to conservative cancer treatment. On the biological level, many pathways such as EGFR-signaling, PI3K/mTOR-pathway and survivin-activation have been associated with chemo- and radioresistance. However, previous studies failed to find an effective treatment against radio- or chemoresistance.

Epigenetic changes, such as histone deacetylation have found to influence response to anticancer treatment. Histone deacetylase inhibitors are used for treatment of many diseases, e.g. valproic acid in epilepsy, but unfortunately results in oncologic therapy have been ambiguous. The group of HDAC inhibitors is a very heterogeneous group with a variety of agents. So far, vorinostat and romidepsin have been FDA approved and recently, it has
been proposed that HDAC inhibitors are a promising group of agents which could enhance the effect of PD-1 antagonists in advanced solid tumors 87.

4.2. Discussion of our Findings

Resminostat is a new pan-HDAC inhibitor which has shown effectiveness in various other tumor cell lines 54.

The first aim of this thesis was to investigate the effect on cell proliferation of resminostat on head and neck squamous cell carcinoma cell lines. Our study shows that it is a very potent agent in treatment of head and neck squamous cell carcinoma with an IC50 lying in the nanomolar to micromolar range. In SCC25, which derive from tongue oral squamous cell carcinoma, induction of apoptosis was significant, whereas it was not significant in in CAL27 and FaDu cells. However, as late apoptosis and necrosis are impossible to be differentiated in our experiments, we analyzed the induction of total cell death by resminostat in our cell lines, which indeed was significant. A control run with a immortalized human skin keratinocyte cell line showed that effectiveness of treatment with resminostat was minimum twice as high in head and neck squamous cell carcinoma cell lines than in the keratinocyte cells and thus indicating a potential specificity for carcinoma cells.

The second aim of this study was to investigate the effect of resminostat on the cell lines in combination with cisplatin treatment, which is a standard therapy in HNSCC. We could prove a strong synergistic effect of both agents. Reduction of cisplatin dosages seems favorable as severe adverse events occur frequently and thus cisplatin is not suitable for all patients 88. However, cumulative dosages of cisplatin and the number of treatment cycles correlate positively with prognosis in HNSCC, and thus reduction of cisplatin dose is not recommended 89. In this study, we could show a synergistic effect with cisplatin treatment in our cells, reducing the IC50 on a nanomolar level. Interestingly, SCC25 cells were most sensitive to resminostat, which are also least sensitive to cisplatin treatment. As this thesis is carried out in cell lines, we can not determine the side effects in as well as prognostic impact of concomitant treatment of cisplatin an resminostat on humans, but however our in-
vitro experiments led to a significant potentiation of cisplatin effect. The experiments of combined treatment with cisplatin and resminostat on protein expression by Western Blot showed a down-regulation of survivin in those cells that had been treated with both agents after 24 hours. Remarkably, the combination of resminostat and cisplatin was more potent in SCC25 and CAL27 cells, which were less sensitive than FaDu cells in our experiments on single treatment with resminostat. This finding supports our theory that resminostat could gain importance to overcome cisplatin resistance. This data also corresponds to the results of previous studies 90,91.

The third aim of this thesis was to investigate the effect of resminostat on irradiated HNSCC cell lines. Overall, a synergistic effect with irradiation was evident. A theoretical study showed that in-vitro assays correspond better to clinical outcome of patients undergoing radiotherapy than other assays 92. However, treatment with resminostat did not show a short-time effect on HNSCC cell lines, but a very effective reduction of clonogenic survival. A possible explanation could be, that its effect is time-dependently enhanced as short-time effect is measured after 3 days in contrast to counting of clones, which is conducted after 10 days 93,94. This is underlined by the time-dependent reduction of survivin expression in those cells receiving single treatment with resminostat. This underlines the potent effect against HNSCC, as previous studies have shown that survivin was associated with a more aggressive phenotype and thus a bad prognostic factor for oral squamous cell carcinoma 95. However, our experiments did only show an effect of survivn down-regulation on CAL27 cells 24 hours after irradiation with 8 Gray. We propose, that resminostat exhibits its full radiosensitizing potential after at least 48 hours-72 hours after radiotherapy. So far, only cetuximab shows a radiosensitizing and chemo sensitizing effect on head and neck squamous cell carcinoma 96 causing a survival- benefit of 20 months 97. But however, there exists a collective of patients who suffer from resistance to cetuximab 98. Thus, we hypothesize, that this could be a potentially eligible patient collective for treatment with resminostat.

As – to our knowledge – no literature about HDAC11 expression in tissue samples of oropharyngeal cancer exists, we performed immunohistochemical staining for HDAC 11 in order to evaluate its expression in oropharyngeal cancer. We could not prove its expression in oropharyngeal cancer. However, we did neither investigate its
existence in other tumor entities than oropharynx nor we investigated its expression in our cell lines.

We did not prove an effect on p-AKT and Mcl-1 expression of resminostat in our cell lines. Brunetto et al. described an interaction with AKT signaling pathway resulting in a down-regulation of BAX and BCL-2 \(^{54}\). However, we could not prove effect on the regulation of p-AKT signaling in our study. Although PI3K-pathway alterations occur on a high number in HNSCC, single PI3K-inhibitors so far have not shown striking effect on head and neck squamous cell carcinoma \(^{61}\). Therefore, we propose that the positive effects of resminostat are mediated by another pathway. Interestingly, survivin, an anti-apoptotic \(^{94,99}\) protein is down-regulated time- as well as dose-dependently. Survivin down-regulation is associated with treatment-effectiveness in HNSCC and thus survivin down-regulation induced by resminostat underlines the positive effect of resminostat on HNSCC cell lines \(^{100}\).

However, as various epigenetic mechanisms exist and so far, not all of them are well investigated, we still do not know enough about potential side effects or non-specific effects of resminostat. A recently conducted phase II trial in Japan showed a safe and potentially survival improving effect for patients with hepatocellular carcinoma in combination with sorafenib \(^{101}\). So far, no in vivo-data of resminostat in HNSCC exists. To conclude, we showed a promising effect of resminostat on head and neck squamous cell carcinoma cell lines, which offers a new therapeutic opportunity for this aggressive disease.
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Elisabeth Foki, M.D.
Department of Otorhinolaryngology, Head and Neck Surgery
Medical University of Vienna
Waehringer Guertel 18-20
A-1090 Vienna
Austria, Europe
Tel.: +43 1 40400 56080
email: elisabeth.enzenhofer@meduniwien.ac.at

Curriculum vitae

Date of birth: 23rd July, 1985
Place of birth: St. Poelten, Austria
Maiden name: Elisabeth Enzenhofer
Marriage: 22nd October, 2016
Nationality: Austria

Education

10/2010 to present
Resident, Medical University of Vienna, Department of Otorhinolaryngology, Head and Neck Surgery

2010 to present
Doctoral program of applied medical sciences “Clinical Experimental Oncology”, Medical University of Vienna, Department of Otorhinolaryngology

2009 to 2010
Research fellow, Department of Medicine II, Division of Cardiology, Medical University of Vienna

08/2009
Promotion to Dr. med. univ., Medical University of Vienna

01/2009
Student exchange Switzerland (Centre hospitalier universitaire vaudois, Lausanne)

10/2007 to 02/2008
Student exchange Switzerland (Centre hospitalier universitaire vaudois, Lausanne)

2003 to 2009
Medical training, Medical University of Vienna

1995 to 2003
Secondary school (Privatgymnasium der Englischen Fraulein, St. Poelten)

Further education

09/2016
59th Annual Meeting of the Austrian Society for Otorhinolaryngology, Head and Neck Surgery, Villach, AT
09/2016  ENT Update Europe, Vienna, AT
09/2015  58th Annual Meeting of the Austrian Society for Oto-Rhinolaryngology, Head and Neck Surgery, Innsbruck, AT
09/2015  Facharztprüfung
05/2015  8. Wörtherseesymposium, Klagenfurt, AT
04/2015  Diploma of the Austrian Medical Chamber in Emergency Medicine, Bad Hofgastein, AT
02/2015  1st International Symposium on Tumor-Host-Interaction in Head and Neck Cancer, Essen, DE
09/2014  58th Annual Meeting of the Austrian Society for Oto-Rhinolaryngology, Head and Neck Surgery, Gmunden, AT
07/2014  50th. Graz Course on Rhinosurgery, Graz, AT
04/2014  6. Wiener Airway-Management Kurs, Vienna, AT
04/2014  Phoniatrie Kurs, St. Virgil, Block II
01/2014  5. Wiener Head and Neck Tage, Vienna, AT
12/2013  Stryker Temporal Bone Dissection Course, Vienna, AT
09/2013  57th Annual Meeting of the Austrian Society for Oto-Rhinolaryngology, Head and Neck Surgery, Graz, AT
03/2013  40th Course on functional and aesthetic Rhinosurgery, Ulm, DE
02/2013  6. Kursus für mikorvaskulären Gewebetransfer im HNO-Bereich, Essen, DE
11/2012  14th Hietzinger HNO Tag, Vienna, AT
09/2012  56th Annual Meeting of the Austrian Society for Oto-Rhinolaryngology, Head and Neck Surgery, St. Poelten, AT
2012    „Teach the Teacher“, Univ. Klinik f. HNO, MUW
12/2011  9th Wullstein-Symposium, Wuerzburg, DE
11/2011  ENT-Update, Mainz, DE
10/2011  Interdisziplinäre Onkologie-HNO, Radioonkologie, Internistische Onkologie, Vienna, AT
09/2011  56th Annual Meeting of the Austrian Society for Oto-Rhinolaryngology, Head and Neck Surgery, Vienna, AT
04/2011  Inhalationsallergie- Epidemiologie und Prophylaxe, Vienna, AT
03/2011  Die Bedeutung des HPV in der HNO, Vienna, AT
01/2011  3. Wiener Airway Management Kurs, Vienna, AT

Scientific Background

10/2010 to present ENT-Department, Medical University Vienna
Group Leader: ao. Univ. Prof. Dr. Dietmar Thurnher

2006 to 2010
Department of Medicine II, Division of Cardiology, Medical University of Vienna
Head: Prof. Dr. Gerald Maurer

Invited speeches

02/2015
"Komplikationen der Tracheotomie" „7. Wiener Airwaymanagementkurs"

Awards & Scholarships

2009
Innovationspreis der Stadt Wien

2008/9
Leistungstipendium der Medizinischen Universität Wien

2008
Topstipendium des Landes Niederösterreich

2005/6
Leistungsstipendium der Medizinischen Universität Wien
**Publications**


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