Published in final edited form as:

Laryngoscope. 2020 November ; 130(11): 2643–2649. doi:10.1002/lary.28441.

Apoptosis Signaling Molecules as Treatment Targets in Head and Neck Squamous Cell Carcinoma

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Abstract

Objectives: To evaluate BCL-2 family signaling molecules in head and neck squamous cell carcinoma (HNSCC) and examine the ability of therapeutic agents with variable mechanisms of action to induce apoptosis in HNSCC cells.

Methods: mRNA expression of *BAK*, *BAX*, *BCL2*, *BCL2L1*, and *MCL1* were measured in The Cancer Genome Atlas (TCGA) head and neck cancer dataset, as well as in a dataset from a cohort at Montefiore Medical Center (MMC). Protein expression was similarly evaluated in a panel of HNSCC cell lines (HN30, HN31, HN5, MDA686LN, UMSCC47). Cell viability and Annexin V assays were used to assess the efficacy and apoptotic potential of a variety of agents (ABT-263 (navitoclax), A-1210477, and Bortezomib).

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Conflicts of Interest: none

Meeting Information:

Work from this manuscript was accepted for podium presentation at the 122nd Annual Meeting of the Triological Society at COSM, Austin, Tx, May 3rd, 2019

Results: Expression of BAK, BAX, BCL2L1, and MCL1 were each significantly higher than expression of BCL2, in the TCGA and MMC datasets. Protein expression demonstrated the same pattern of expression when examined in HNSCC cell lines. Treatment with combined ABT-263 (navitoclax) / A-1210477, or with bortezomib demonstrated apoptosis responses that approached or exceeded treatment with staurosporine control.

Conclusion: HNSCC cells rely on inhibition of apoptosis via BCL-xL and MCL-1 over-expression, and induction of apoptosis remains a potential therapeutic option, as long as strategies overcome redundant anti-apoptotic signals.

Keywords

Head and Neck Squamous Carcinoma; Apoptosis; ABT-263; A-1210477; Bortezomib

Introduction

Resisting cell death via evasion of apoptosis has been deemed a hallmark of cancer^{1,2}, and the survival of head and neck squamous cell cancer (HNSCC) depends on this hallmark. The upper aerodigestive tract squamous epithelium forms a stratified layer of cells that under normal circumstances terminally differentiate and die in an organized process that relies on several apoptosis signaling molecules³. It is not surprising that dysregulation of apoptosis is a critical component of the carcinogenesis process in HNSCC⁴. Indeed, *TP53* is the most common mutation in HNSCC⁵⁻⁷, and a canonical model of p53 tumor suppression is to induce apoptosis in response to cell stressors such as DNA damage or activated oncogenes⁸.

Other genes involved in the intrinsic pathway of apoptosis, such as anti-apoptotic molecules *BCL2*, *BCL2L1* (coding for the BCLxL protein), and *MCL1*, as well as pro-apoptotic *BAX* and *BAK*, appear to remain intact in HNSCC⁵. As one means to evade apoptosis, cancer cells commonly upregulate BCL-2 family pro-survival molecules⁹, and several studies have examined expression of these molecules and their role in treatment resistance in HNSCC¹⁰⁻¹². Recent work by our group has suggested that the pro-survival BCL-xL and MCL-1 proteins are frequently overexpressed in HNSCC, and we demonstrated that combined targeting of these two proteins resulted in synergistic effects on cell viability *in vitro*¹³.

It appears that HNSCC depends on disruption of the normal apoptosis response, yet the intrinsic machinery remains functional. If true, activation of apoptosis could potentially be exploited in HNSCC, yielding an effective treatment strategy in HNSCC. In the current study, we confirm that BCL-xL and MCL-1 are expressed at high levels in HNSCC, along with pro-apoptotic BAX and BAK. Our previous publication demonstrated that inhibition of BCL-xL and MCL-1 resulted in synergistic effects on cell viability¹³. Here, we examine the apoptosis response using Annexin V assays after targeting HNSCC cells with several agents that alter apoptosis signaling - specifically treatment with the BCL2/BCL-xL inhibitor ABT-263 (navitoclax), the MCL-1 inhibitor A-1210477, and the proteasome inhibitor, Bortezomib.

Methods:

Gene Expression Microarray and RNAseq Data

Data from a total of 496 HNSCC patients in The Cancer Genome Atlas (TCGA) cohort include level 3 RNA sequencing data (log₂ transformed RSEM value, as reads per kilobase of transcript per million mapped reads) and clinical data (HPV status, tumor stage, and primary tumor site) downloaded from NCI Genomic Data Commons (GDC, <https://portal.gdc.cancer.gov/>). Relative gene expression levels of targeted genes were presented as box-plots using GraphPad Prism® v. 8 (San Diego, CA). In parallel, the relative gene expression levels were examined from the targeted genes within tumor samples obtained and evaluated at Montefiore Medical Center (MMC). Ribonucleic acids (RNA) from HNSCC tumors were hybridized to the Illumina® HumanHT-12-v3 Expression BeadChip (Illumina®, San Diego, CA) and data were normalized and evaluated, as described in previous reports^{13–15}. For this analysis, evaluation was restricted to tumors from patients with histologically-proven HNSCC, without previous treatment, and without a history of a previous HNSCC primary tumor. Gene expression data from 131 HNSCC patients was log₂ transformed, analyzed, and presented as box plots using GraphPad Prism® v. 8 (San Diego, CA).

Pairwise Pearson correlation analysis was conducted with relative gene expression levels of *BAX*, *BAK1*, *BCL2*, *BCL2L1*, and *MCL1* within TCGA and MMC cohorts using log₂ transformed data. A correlation coefficient matrix was produced by combining pairwise correlation coefficients from TCGA and MMC data, respectively. The matrix was used for unsupervised hierarchical clustering using the Pheatmap package applied in R® statistical software.

HNSCC cell culture

Cell lines HN30, HN31, HN5, MDA686LN, and UMSCC47, were graciously provided by Dr. Jeffrey N. Myers, MD, PhD at the University of Texas, MD Anderson Cancer Center. Transfer agreements required the following permissions (HN30, HN31 – John Ensley, MD, Wayne State University; MDA686LN – Peter Sacks, MD, New York University/University of Texas MD Anderson Cancer Center; UMSCC47 – Thomas Carey, University of Michigan). All cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) combined with 10% Fetal Bovine Serum (FBS), nonessential amino acids, sodium pyruvate and 1% antibiotic — penicillin/streptomycin. Incubation was maintained at 37°C, 5% CO₂. Stocks of each line used were authenticated with short tandem repeat (STR) genotyping.

Western blot analysis

Whole cell lysates from HSNCC cells were prepared in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor cocktail (Pierce™, Thermo Scientific, Rockford, IL, USA). Protein concentrations were measured using DC™ protein assay (Bio-Rad™, Hercules, CA, USA). Sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) was run at 80V and membrane immunoblotted overnight. Primary antibodies were incubated in 1% bovine serum albumin (BSA)/Tris-buffered saline with 0.1% Tween-20 at concentrations varying from 1:500 – 1:5000 (anti-β-actin – 8H10D10, 1:5000; anti-BCL-2

(124) 15071S, 1:500; anti-mcl-1 (D35A5) 5453S; anti-bax 2772S, 1:500; anti-BCL-xL (54H6) 2764s, Cell Signaling, 1:500; each from Cell Signaling, Danvers, MA, USA; and anti-BAK, 1:500, NT, EMD Millipore Corp., Billerica, MA, USA. Fluorescent secondary antibodies against mouse or rabbit were sourced from LI-CORTM (Lincoln, NE, USA). Immunolabeled proteins were visualized with the LI-COR[®] Odyssey[®] FC Imaging System. Protein quantification was determined from 3 independent experiments using image studio software (LI-COR[®] Biosciences, Lincoln, NE).

Cell Viability Assays - Screening

A pipetting robot was utilized to plate cells and test agents in a rapid, high throughput fashion. HNSCC cells (1500 cells/well) were seeded in 96-well white plates. Cells were incubated with serial dilutions of Bortezomib. Control (untreated) wells were maintained in parallel dilutions of dissolvent DMSO. Cell Titer-Glo (Promega, Madison, WI), was used to assess cell viability according to the manufacturer's protocol. The TECAN M200 microplate reader was used to measure luminescence. Viability assays were performed in duplicates and the data normalized to vehicle-treated control wells. IC50 values were determined by nonlinear regression analysis using Prism software (GraphPad Software, La Jolla, CA).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Viability Assay
HNSCC cell lines were plated on 96-well plates at a density of 1,500 cells/well. Cells were allowed to incubate for 24 hours before addition of bortezomib, with doses ranging from 0.31nM – 160nM. After 48hr drug incubation, 10µl of MTT (VWR Chemicals, Solon, Ohio, USA) at 5mg/ml was added until visualization of formazan crystals by 150 minutes. Cell viability was determined by spectrophotometry at 570nM. IC50 values were calculated by non-linear regression analysis of the recorded absorbances with respect to vehicle control wells using GraphPad Prism[®] v. 8 (San Diego, CA).

Bortezomib treatment of HNSCC

HNSCC cells were plated onto 10cm plates and allowed to grow for 24h. The following day, DMEM containing 5nM or 10nM Bortezomib (EMD Millipore Corp, USA) was added and incubated for 24h at 37°C with 5% CO₂. Whole cell lysate was extracted in RIPA buffer at the end of incubation.

Annexin V Apoptosis Assay

125,000 HNSCC cells were seeded in each well of 6 well plates and incubated for 24 hours. After 24hrs, HNSCC were treated with apoptosis inducing agents at the following concentrations: 1µM Staurosporine (Adipogen Life Sciences, San Diego, CA), 5µM ABT-263 (navitoclax) (Selleck Chem, Houston, TX), 5µM A-1210477 (Selleck Chem, Houston, TX), and 5µM ABT-263 (navitoclax)/5 µM A-1210477 combination 5nM Bortezomib, and 10nM Bortezomib. Cells were incubated for another 48hours and harvested with accutase, washed in PBS and stained with PE Annexin V Apoptosis Detection Kit I (BD Biosciences, San Diego, CA). Analysis was conducted by flow analyzer (BD LSR II Yellow). Flow cytometer data was analyzed and figures produced by FlowJoTM v10 software (FlowJo, LLC, Ashland, OR).

Statistical Analyses

Mean averages were calculated for each transcript assessed from gene expression data in TCGA and MMC datasets, and transcripts were compared against each other using two-tailed Student T-tests. Median or mean averages were calculated where appropriate for Annexin V data and for protein quantification, and box-plots were created to represent data, where described. For statistical analyses, a p-value of 0.05 was accepted as the threshold for significance. Statistical analyses for specific assays are included in more detail in individual sections above.

Results

Evaluation of Expression of BCL-2 Family Transcripts in TCGA and MMC HNSCC

The relative gene expression of *BCL2*, *BCL2L1*, *MCL1*, *BAX*, and *BAK1* were evaluated in TCGA dataset (Figure 1A) and the MMC dataset (Figure 1B). Both datasets demonstrated a similar pattern of expression, and expression of pro-apoptotic *BAX* and *BAK1*, as well as anti-apoptotic *BCL2L1* and *MCL1* were all significantly higher than expression of *BCL2* based on comparisons with Student T-tests in both datasets. Correlation matrices for these transcripts were generated for TCGA (Figure 1C) and MMC data (Figure 1D) in an attempt to examine the balance of pro- and anti-apoptotic transcripts. In both TCGA and MMC datasets the only strong anti-correlation was noted between *BCL2* and *BAK1*.

Expression of BCL Family Proteins in HNSCC Cell Lines

HNSCC cell lines HN30, HN31, HN5, MDA686LN, and UMSCC47 were used for further studies. These lines represent HNSCC cells from a variety of different HNSCC sites (Table 1), and included a cell line known to carry wildtype *TP53* (HN30), its isogenic line derived from a lymph node metastasis harboring mutant copies of *TP53* (HN31), as well as an HPV-positive cell line (UMSCC47)¹⁶. Baseline protein expression of BCL-2, BCL-xL, MCL1, BAX, and BAK were evaluated (Figure 2A) and average values after quantification of these proteins from multiple blots demonstrated a pattern of expression similar to gene transcription seen in the patient cohorts, with low BCL2 expression, and relatively higher expression of BCL-xL, MCL1, BAX, and BAK (Figure 2B).

Apoptosis Response After Targeting BCL-xL and MCL1

Since it was apparent that HNSCC expresses both pro- and anti-apoptotic signaling molecules, several approaches of targeting apoptosis were evaluated. Data presented above suggests that multiple redundant mechanisms are in place for HNSCC to resist apoptosis. For this reason, strategies that alter multiple anti-apoptosis mechanisms were chosen for further evaluation. Our previous work demonstrated that concurrently targeting BCL2, BCL-xL and MCL-1 resulted in synergistic reduction in cell viability in multiple HNSCC cell lines¹³. In the current study, apoptosis was evaluated in the cell line panel evaluating Annexin V after treatment with ABT-263 (Navitoclax), after treatment with the MCL-1 inhibitor, A-1210477, and after combination treatment. Combination treatment significantly increased apoptosis compared to single-agent treatment in all cell lines, and reached levels

near or greater than samples treated with the control apoptosis agent staurosporine (Figure 3).

Cell Viability and Apoptosis Response After Bortezomib Treatment

Bortezomib has previously been shown to induce apoptosis via several mechanisms¹⁷, such as inducing the unfolded protein response¹⁸, and via alterations in the levels and activity of apoptosis signaling molecules^{19,20}. Therefore, the cell viability and apoptosis response to bortezomib was evaluated in our HNSCC cell line panel. Bortezomib showed exquisite responses on drug screening (supplemental Figure 1), and responses were validated using MTT assays. Results demonstrated IC50 doses ranging from 1.6 – 10.15 nM, confirming that HNSCC cells are highly sensitive to Bortezomib (supplemental Table 1, and Figure 4). When apoptosis responses were evaluated with Annexin V assays, HNSCC cell lines each demonstrated a dose-dependent response to Bortezomib, with responses nearing or exceeding staurosporine controls at 10nM of Bortezomib in HN30, HN31, and HN5 (Figure 5). Protein levels of BCL2, BCL-xL, MCL1, BAX, and BAK were evaluated by western blot before and after Bortezomib treatment, however there were no significant or consistent changes noted in the overall protein expression of these factors (Supplemental Figure 2).

Discussion

In this report, we provide evidence that BCL-xL and MCL-1 play an important role in the mechanism by which HNSCC tumors resist apoptosis. We also demonstrate that activation of apoptosis remains available as a treatment strategy in HNSCC as long as one can overcome redundant mechanisms that keep apoptosis in check in this disease. Anti-apoptotic *BCL2L1* and *MCL1* transcripts were highly expressed in the TCGA dataset, as well as in an archival dataset from our institution. These same proteins were elevated in HNSCC cell lines. Pro-apoptotic BAX and BAK were also expressed in patient samples and HNSCC cell lines. This expression pattern suggests that targeting apoptosis via inhibition of both BCL-xL and MCL1 would be effective and potentially synergistic, which is what was observed in HNSCC lines in our recent publication¹³. In this current follow-up report, we show that combination ABT-263 (navitoclax) plus A-1210477, collectively inhibiting BCL-xL, BCL2, and MCL1, results in a strong apoptosis response. The redundancy in anti-apoptotic signaling also provides some rationale for the efficacy of bortezomib, which potentially activates apoptosis via several pathways as sequelae after proteasome inhibition.

BCL2 family proteins have been examined previously in HNSCC. Work by Michaud and colleagues suggests that BCL2 over-expression is associated with treatment failure and cisplatin resistance in HNSCC cell lines, whereas BCL-xL expression showed no such correlation¹¹. Alternatively, Bauer and colleagues examined BCL-xL expression among the Department of Veteran's Affairs Laryngeal Cancer Study, and found that high BCL-xL expression was associated with a low rate of laryngeal preservation¹⁰. Whether these markers are truly prognostic remains an open question, and our study did not focus on the utility of BCL2 family proteins as prognostic biomarkers. Our data suggest that high expression of BCL-xL is perhaps a hallmark of HNSCC in general, and thus may not be a good prognostic marker. However, BCL-xL may certainly be a valid treatment target. We

also demonstrate that targeting BCL-xL is much more effective when combined with MCL-1 inhibition, resulting in substantially increased apoptosis, supporting cell viability results from our previous study¹³.

Targeting BCL-2 family proteins has been an area of research in HNSCC, and as newer agents targeting apoptosis are developed, exploration in this field has expanded. Li and colleagues published proof-of-concept work that demonstrated efficacy of targeting BCL2 and BCL-xL using cell permeable peptides derived from the BH3 domains of Bad, Bak, and Bax²¹. The *in vitro* efficacy of the first-generation BCL2/BCL-xL inhibitor, ABT-737, has been examined in HNSCC cells in combination with cisplatin and etoposide²², and also with sorafenib²³. Other apoptosis-acting therapeutics, including AT-101²⁴, obatoclax²⁵, and venetoclax²⁶, have been studied as single agents. Several studies, prior to the advent of newer MCL1-directed molecular agents, have examined down-regulation of MCL1 as an effective mechanism of action of several drugs in HNSCC. Early *in vitro* work studying HNSCC work showed that inhibition of MCL1 using antisense oligonucleotides could enhance the efficacy of several chemotherapeutic agents²⁷. Zhang et al. studied the proteasome inhibitor carfilzomib in HNSCC and found that upregulation of MCL1 led to resistance to this therapy, while inhibition of MCL-1 with si-RNA resulted in improved responses²⁸. The epidermal growth factor receptor (EGFR) inhibitor, afatinib, has also been shown to down-regulate MCL-1 and induce apoptosis in HNSCC²⁹. These studies support the importance of MCL-1 in inhibiting apoptosis and resisting standard treatments in HNSCC.

MCL-1-specific inhibitors have been developed recently, and we are the first to our knowledge to report activity of A-1210477 in HNSCC, both in a recent report¹³ and this current study. We demonstrate that combining MCL-1 inhibition with BCL-2/BCL-xL/BCL-w inhibition via ABT-263 (navitoclax) results in a substantial increase in apoptosis across multiple HNSCC cell lines. Though targeting of specific BCL-family molecules may be efficacious in certain malignancies, such as targeting BCL-2 with venetoclax in chronic lymphocytic leukemia³⁰, it seems that combination therapy targeting multiple BCL-family proteins may be necessary in most solid tumors. Inoue-Yamauchi and colleagues recently demonstrated that indirect downregulation of MCL-1 with either anthracyclines or CDK9 inhibition was necessary to overcome resistance to ABT-263 in small cell lung cancer cells³¹. In 2015, Maji et al. demonstrated that MCL-1 knockdown improved response of oral cavity squamous cell cancer cells to the BCL-2/BCL-xL inhibitor ABT-737, and they showed that the pan- anti-apoptotic inhibitor sabutoclax was effective in a murine model as a single agent³². Another recent study demonstrated that mantle cell lymphomas that resist treatment with BCL-2 specific venetoclax can be overcome with the synthetically lethal combination of venetoclax and the MCL-1 inhibitor S63845³³.

In this study, we describe bortezomib as an agent that induces apoptosis in HNSCC. As stated previously, bortezomib activates apoptosis via the unfolded protein response and ER stress following proteasome inhibition^{18,34}. Additionally, bortezomib may alter the balance of BCL2 family apoptosis signaling molecules, several of which are regulated by proteosomal degradation¹⁷. Bortezomib has received significant attention in HNSCC. Li and colleagues demonstrated that bortezomib could activate apoptosis and synergize with

cisplatin in HNSCC²⁰. Other studies have suggested that bortezomib can liberate p53, contributing to apoptosis in HPV-positive HNSCC^{20,35}. Chen and colleagues were among the first to examine bortezomib in HNSCC cells, and also demonstrated *in vitro* activity at nanomolar ranges³⁶. Unfortunately, promising preclinical data have not translated into clinical activity using bortezomib. Bortezomib was examined alone and in combination with irinotecan in HNSCC patients in a large, multi- institutional phase-II trial supported by the Eastern Cooperative Group (ECOG E1304), with discouraging results³⁷. Though bortezomib is approved for the treatment of multiple myeloma, the drug has not found utility in solid tumors. It is unclear whether this is due to limited activity of the drug, the bioavailability and pharmacokinetics of the agent³⁸, or inherent resistance factors within tumors. Nevertheless, our study showed that bortezomib could induce apoptosis in HNSCC at very low doses, and we speculate that this may be secondary to the drug's ability to overcome several redundant apoptosis resistance mechanisms in HNSCC.

Conclusions

In summary, though evasion of apoptosis is necessary for cancer cell survival, HNSCC cells maintain intact apoptosis machinery, and appear to rely on inhibition of apoptosis via BCL-xL and MCL-1 over-expression. Therefore, induction of apoptosis remains a potential therapeutic option, as long as strategies overcome redundant anti-apoptotic signals inherent to HNSCC cancer cell survival. Our work studying the activity of ABT-263, A-1210477, and bortezomib, support this concept.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding:

Thomas J. Ow's contribution was supported in part by the Triological Society Career Development Award, NIH-NCI grant 2K12 CA132783-06, and NIH-NIDCR grant 1 K23 DE027425-01. E.G. and D.E.R. were supported from NIH-NCI grant CA178394 and an award from the Pershing Square Sohn Cancer Research Alliance. AL was supported from a T32 Training grant T32 AG 23475. The authors also acknowledge support from the Albert Einstein Cancer Center (NIH-NCI P30CA013330), and specifically the flow cytometry core facility, part of the Albert Einstein shared resources, as well as Roswell Park Comprehensive Cancer Center (CA016056). The manuscript content is solely the responsibility of the authors and do not necessarily represent the official views of the National Institute of Health (NIH).

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Figure 1.

Relative gene expression levels of *Bax* (green), *Bak1* (salmon), *Bcl2* (red), *Bcl2l1* (blue), and *Mcl1* (purple) of the HNSCC cases within TCGA (A) and MMC (B) cohorts, respectively. The horizontal middle line is the median; the height of the boxes indicate the interquartile range (IQR) with top and bottom marking the 25 and 75 percentiles, respectively; dots mark outliers. The bottom panel heatmap shows pairwise correlation coefficients of relative gene expression levels of *Bax*, *Bak1*, *Bcl2*, *Bcl2l1*, and *Mcl1* of the HNSCC cases within TCGA (C) and MMC (D) cohorts, respectively. Red denotes a positive correlation and blue denotes a negative correlation.

Figure 2. Representative western blot data demonstrating protein expression of *BAX*, *BAK*, *BCL2*, *BCL-xL*, and *MCL-1* in HNSCC cell lines (HN5, HN30, HN31, MDA686LN, and UM5CC47) (A). Quantification of protein bands normalized to β -Actin loading controls based on replicated data for each cell line, and pooled across all lines examined. The horizontal middle line is the median fold expression; the height of the box indicates the interquartile range (IQR) (B).

Figure 3.

Apoptosis response in HNSCC cell lines based on Annexin V assays after treatment with staurosporine as a control showing, ABT-263 (navitoclax), A-1210477, and combination ABT-263 (navitoclax)/A-1210477. Data are presented for each cell line (A.), and pooled (B.), where the horizontal line represents the mean value for all assays and the whiskers represent the standard deviation.

Figure 4.

Representative dose-response curves after bortezomib treatment for HNSCC cell lines HN30 (A), HN31 (B), HN5 (C), MDA686LN (D), and UMSCC47 (E).

Figure 5.

Apoptosis response in HNSCC cell lines based on Annexin V assays after treatment with staurosporine as a positive control, and Bortezomib at 5nM and 10 nM. Data are presented for each cell line (A), and pooled (B), where the horizontal line represents the mean value for all assays and the whiskers represent the standard deviation.

Table 1.

Head and Neck Cancer Cell Line Characteristics

Cell Line	Site	HPV Status
HN30 *	Hypopharynx	Negative
HN31 *	Lymph node metastasis	Negative
HN5	Oral Cavity	Negative
MDA686LN	Lymph node metastasis (Oropharynx)	Negative
UMSCC47	Oral Cavity	Positive

*
isogenic cell lines

Table 2.

IC50 values for Bortezomib in HNSCC cell lines

Cell line	IC50 (nM)	SD (nM)
HN30	1.62	±0.74
HN31	3.45	±0.64
HN 5	10	±0.57
MDA686LN	10.15	±1.63
UMSCC47	10.15	±6.72

SD = standard deviation

nM = Nanomolar

HNSCC = Head and neck squamous cell carcinoma