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IN VITRO ANTICANCER ACTIVITY OF HISTATIN-1 COMBINATION WITH CISPLATIN IN HEAD AND NECK CANCER CELL LINES

Background. Chemotherapy of head and neck squamous cell carcinoma (HNSCC) is associated with significant side effects. Antimicrobial peptides (AMPs), which are naturally occurring defense molecules like defensin-1 and LL-37 found in human secretions, have demonstrated potential in prompting tumor cell apoptosis and enhancing the effect of chemotherapeutic agents. However, the anticancer potential of histatin has not yet been thoroughly examined. The **aim** of the study was to explore the anticancer activity of histatin, an AMP present in human saliva and used alone or in combination with cisplatin in HNSCC cell lines. **Materials and Methods**. The gene expression of histatin was evaluated in the HSC4 and SCC25 cell lines by qRT-PCR. Cell proliferation was investigated at different concentrations of histatin peptide (His-1), cisplatin, and their combination using an MTT assay. **Results**. SCC25 cells expressed both *HTN1* (histatin-1) and *HTN3* (histatin-3), whereas the HSC4 cell line expressed only *HTN1*. The combination of exogenous His-1 and cisplatin demonstrated a synergistic anti-proliferative effect against the HNSCC cell lines in a dose-dependent manner. **Conclusions**. The combination of low-dose cisplatin and histatin inhibits HNSCC cell proliferation. His-1 sensitizes tumor cells to the cytotoxic effects of cisplatin potentially allowing for a reduction in its effective concentration.

Keywords: AMPs, anticancer activity, combination therapy, histatin, HNSCC.

GLOBOCAN 2020 reported head and neck squamous cell carcinoma (HNSCC) as the 6th most common cancer representing approximately 4.2% of all cancers worldwide [1]. Oral squamous cell carcinoma (OSCC) is one of the most prevalent malignant diseases and a subtype of HNSCC, originating from the oral mucosa [2, 3]. Despite ongoing advancements in the strategies for cancer detection and treatment, the survival rates of patients with OSCC continue to be discouraging [4]. Addressing this challenge, there is a growing interest in developing new therapeutic approaches that target molecular mechanisms regulating tumor cell growth or cell death, with the ultimate goal of improving patients' survival and quality of life.

Chemotherapy is one of the most effective and widely used approaches to treat HNSCC. It is known to induce apoptosis in cancer cells [3]. Similar to other solid tumors, oral cancer poses chal-

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lenges to chemotherapy effectiveness and patient survival, primarily due to the emergence of drug resistance and the potential toxicity to healthy cells [5]. This results in dose-dependent adverse effects in the patients, including fatigue, weakness, nausea, hair loss, vomiting, and, in severe cases, death [1]. Exploring novel and more effective drugs, along with employing combination chemotherapy, emerges as a promising solution to these challenges. The recent research has spotlighted potential synergistic combinations against oral cancer in both *in vitro* and *in vivo* settings, incorporating various drugs like cisplatin, 5-fluorouracil, carboplatin, and paclitaxel, as well as natural compounds such as resveratrol and curcumin [6].

Antimicrobial peptides (AMPs) are small peptides widely found in natural sources including human secretions. AMPs represent a new class of anticancer drugs that lack toxicity and may overcome tumor resistance to conventional chemotherapy [2, 7]. The AMP stability in biological fluids and anticancer efficacy could be enhanced by different modifications introduced by chemical synthesis [2]. AMPs such as defensins, cathelicidin (LL-37), and magainin-2 have demonstrated anti-metastatic, antiproliferative, and immunomodulatory properties, and they enhanced the efficacy of chemotherapy via selective targeting of cancer cells [8]. The cationic AMPs can disrupt microbial cell membranes by interacting with negatively charged phospholipids. Moreover, several peptides are capable of triggering cytotoxicity of human cancer cells by binding to negatively charged phosphatidylserine moieties, which are selectively exposed on the outer surface of cancer cell plasma membranes [2].

Histatin is a histidine-rich cationic AMP consisting of 38 amino acids found in the saliva of higher primates [9]. It plays a protective role in saliva and contributes to digestion, lubrication, protection, and immune defense of the oral cavity [10]. Emerging research suggests that histatins may exert notable effects on cells beyond antimicrobial ones. Histatins have been reported to influence cellular proliferation, differentiation, and wound-healing processes and modulate cell signaling pathways, potentially impacting cell growth [11, 12]. Moreover, histatins, through their interaction with cellular membranes, may contribute to cellular adhesion and migration, suggesting a role in tissue repair and regeneration [13]. These multifaceted cellular effects underscore the complexity of histatin function and prompt further investigation of their potential applications in cancer treatment.

The combination of AMPs and chemotherapy drugs has shown better results. For instance, when LL-37 was combined with etoposide, it exerted a significantly enhanced antitumor effect on glioma cells [14]. This synergism not only enhances the efficacy of traditional anticancer drugs but also reduces the required dosage of the peptide, thereby lowering potential cytotoxicity. Despite the limited research on the anticancer properties of histatin [15], particularly in the context of combination therapy, it presents an area ripe for further exploration. To address this gap, our study investigates the anticancer activity of exogenous histatin-1 (His-1), cisplatin, and their combination against HNSCC cells *in vitro*.

Materials and Methods

Human HNSCC cell lines. The human head and neck squamous cancer cell line HSC4 was obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB Cell Bank, Osaka, Japan), and SCC25 and HaCaT, the human immortalized keratinocyte cell line, were acquired from the American Type Culture Collection (ATCC, USA). All cell lines were cultured in a 1:1 mixture of Dulbecco's Modified Eagle's medium (DMEM) (Invitrogen. ThermoFisher, USA) and nutrient mixture Ham's F12 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA). Both media were supplemented with antibiotics (penicillin and streptomycin) (Invitrogen, USA) and an antifungal drug (amphotericin B 250 μg/mL) (Invitrogen, USA).

Reagents. Human salivary histatin-1 (His-1) was chemically synthesized and obtained from the Bio Basic, Canada. The acquired sequence DSpHEKRHHGYRRKFHEKHHSHREFPFYGDYGSNYLYDN [16] corresponded to the 38 amino acid length phosphopeptide for His-1 with 98% purity. Cisplatin (MedChemExpress, USA) and His-1 were dissolved in DMSO (1:1000) to create a stock solution of 100 mM stored at –20 °C. Both cisplatin and His-1 were then diluted to concentrations of 0.3 μM, 1 μM, 3 μM, 10 μM, and 30 μM. The concentrations for the His-1 and cisplatin combination were determined based on the respective IC_{50}

values in the HSC4, SCC25, and HaCaT cell lines. His-1 was diluted to different concentrations of 2.5 μM, 5 μM, 10 μM, 20 μM, and 40 μM, while cisplatin was diluted to concentrations of 0.5 μM and 1 μM. All cell lines were treated with all possible two-drug combinations involving histatin and cisplatin.

Real-time polymerase chain reaction (qRT-PCR). HSC4, SCC25, and HaCaT cells were cultured in 35-mm culture plates until 90% confluence. Total mRNA was isolated from the cells using an RNA isolation kit, PureLink® RNA Mini Kit (Invitrogen, USA). The yielded RNA was quantified at an absorbance of 260/280 nm using a Nanodrop spectrophotometer (ThermoFisher, USA). cDNA was synthesized from isolated RNA using SuperScript™ IV VILO™ Master Mix (Invitrogen, USA) according to the manufacturer's instruction. The quality and amount of both mRNA and cDNA were assessed using a Nanodrop spectrophotometer. The forward and reverse primer sequences are presented in Table 1. The 18S ribosomal RNA gene was used as a housekeeping gene. The relative mRNA gene expression of the studied genes was calculated by the $2^{-\Delta\Delta CT}$ method.

Cell viability assay (MTT assay). Cell viability was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. All cell lines were seeded in sterile 96-well bottom plates, 100 μL per well (SPL Life Sciences, Korea), and treated with cisplatin, His-1, or their combination for 24 and 48 h. After the incubation, 0.5 mg/mL of MTT reagent (Invitrogen, USA) was added to each well, and the plates were kept in the dark for 4 h. The MTT-containing media was removed, and the remaining purple formazan crystals were dissolved in 100 μL of dimethyl sulfoxide (DMSO). The absorbance values were read out at 570 nM using a microplate reader. Data were expressed in relation to the control.

Statistical analysis was performed using the statistical software SPSS Version. Gene expressions in the cell lines were compared using Student's *t*-test and the cell proliferation — using the one-way ANOVA with multiple comparisons. *p* value < 0.05 was considered statistically significant.

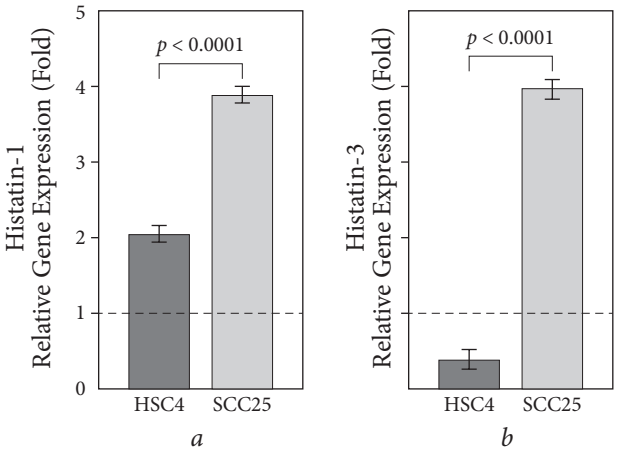


Fig. 1. The relative gene expression of human histatins in HSC4 and SCC25 cell lines. The expression was normalized by 18S RNA. Data are presented as mean ± SD from 3 independently experiments each performed in triplicate. The gene expression in HaCaT cells served as a baseline (dash line): *a* — histatin-1; *b* — histatin-3

Results

Differential expression of HTN1 and HTN3 in the HNSCC cell lines. First of all, we quantified the endogenous levels of the *HTN1* (His-1) and *HTN3* (His-3) genes expressions using quantitative RT-PCR in the HNSCC cell lines. The gene expression in HaCaT cells, immortalized normal keratinocytes, served as he baseline. 18S rRNA was used as a housekeeping gene for the relative gene expression normalization. The relative expression of the *HTN1* and *HTN* genes in the HSC4 and SCC25 cells is shown in Fig. 1, *a* and 1, *b*, respectively. Both HSC4 and SCC25 expressed the *HTN1* gene at significantly higher levels than normal keratinocytes (2 and 4 folds, respectively). HSC4 cells expressed the *HTN3* gene nearly at a 0.4-fold lower level than HaCaT cells while SCC25 expressed the *HTN3* gene at a nearly 4-fold higher level than normal keratinocytes.

List of the primers for qRT-PCR

Primer	Forward primer	Reverse primer
Histatin-1	5'CGCTGATTCACATGAAAAGAGAC-3'	5'AGGGAAGTATCATGAAACACAGA-3'
Histatin-3	5'CAGTTCGAGTAGCACTGACTAT-3'	5'TCTAGATGTGATTGAGGACCAT-3'
18S rRNA	5'ACCCGTTGAACCCCATTCGTGA-3'	5'GCCTCACTAAACCATCCAATCGG-3'

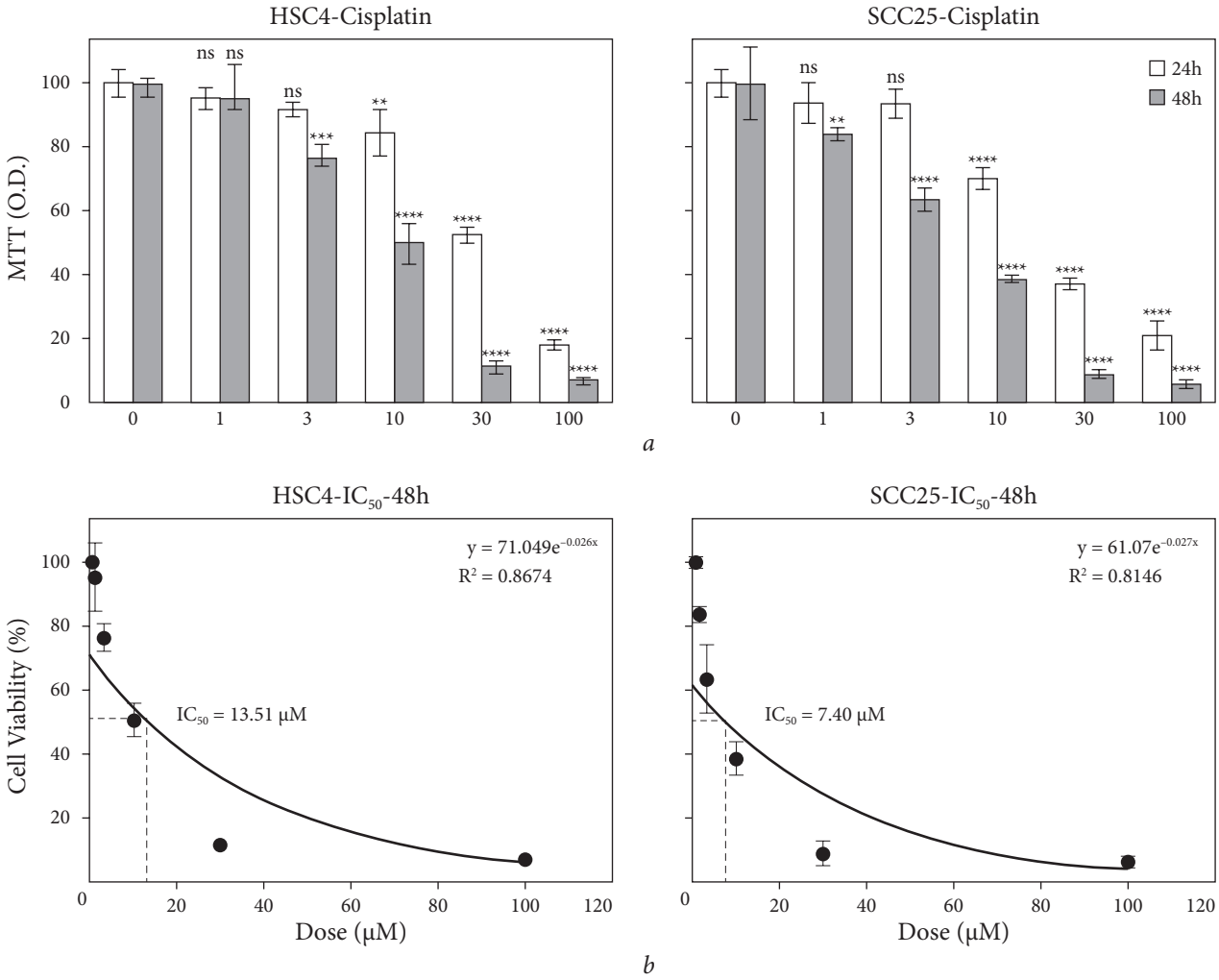


Fig. 2. The growth inhibitory effects of cisplatin in HNSCC cells. HSC4 and SCC25 were treated with cisplatin for 24 h and 48 h. Cell proliferation was evaluated using an MTT assay. Each experiment was performed in triplicate, and data are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ compared to control without cisplatin (*a*). Dose-response curve of cisplatin treatment of HSC4 and SCC25 for 48 h (*b*). The half-maximal inhibitory concentration (IC_{50}) of cisplatin was estimated

Cisplatin decreases the viability of HNSCC cells at high concentrations. Cellular metabolic activity was assessed by quantifying the activity of the mitochondrial enzyme succinate dehydrogenase through the MTT assay. The growth inhibitory effects of 24-h and 48-h treatment with cisplatin were only assessed in the HSC4 and SCC25 cell lines and demonstrated a concentration-dependent decrease in cell viability (Fig. 2, *a*). The half-maximal inhibitory concentrations (IC_{50}) of cisplatin for the HSC4 and SCC25 cells at 48 h incubation period were calculated from dose-response curves (Fig. 2, *b*) and corresponded to 13.51 μM and 7.40 μM, respectively.

Cytotoxic effect of histatin-1 peptide on HNSCC cells. To evaluate the effect of exogenous His-1 on the proliferation of HNSCC cells, we added synthesized His-1 peptide at varying concentrations (1—100 μM) to cultured HSC4 and SCC25 cells for 24 and 48 h and analyzed their viability (Fig. 3). At concentrations below 10 μM, His-1 exhibited minimal impact on the viability of HSC4 or SCC25 cells, but in the range of 10—100 μM, demonstrated dose-dependent inhibition of proliferation, especially after 48 h treatment. When His-1 was added at concentrations higher than 30 μM, we observed more than 50% inhibition in both cell lines (Fig. 3, *a*). The IC_{50} values for His-1 were equal to 38.54 μM and 34.88 μM in HSC4 and SCC25 cells respectively (Fig. 3, *b*). Despite a significant difference in the *HTN1* gene expression levels between these cell lines, there appears to be no sub-

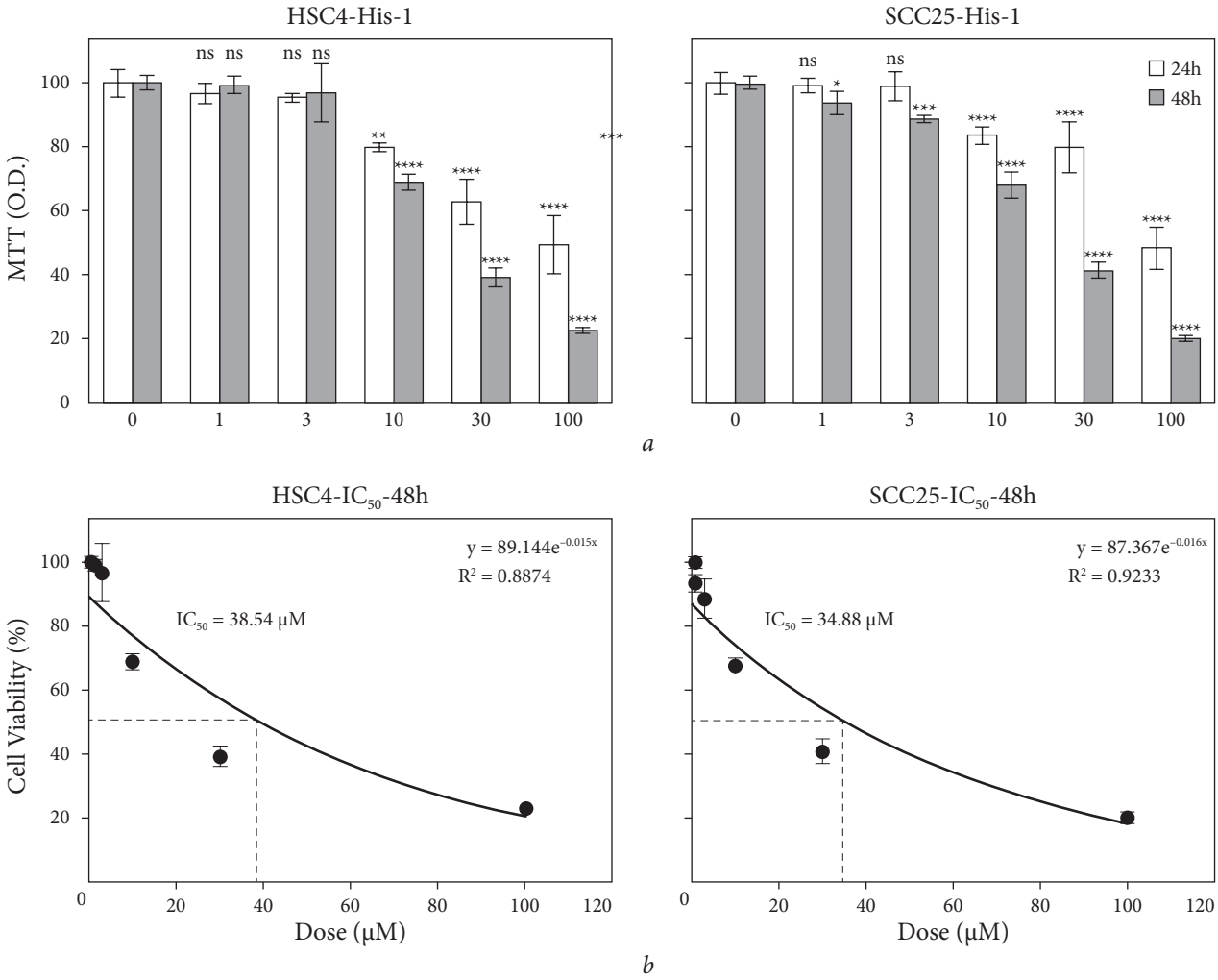


Fig. 3. The growth inhibitory effects of His-1 in HNSCC cells. HSC4 and SCC25 were treated with His-1 for 24 h and 48 h. Cell proliferation was evaluated using an MTT assay. Each experiment was performed in triplicate, and data are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ compared to control without His-1 (*a*). Dose-response curve of histatin treatment of HSC4 and SCC25 for 48 h (*b*). The half-maximal inhibitory concentration (IC_{50}) of His-1 was estimated

stantial difference in the inhibitory effect of His-1 (Fig. 3, *b*). It is noteworthy that the estimated IC_{50} in HaCaT was about 50 μM (data not shown).

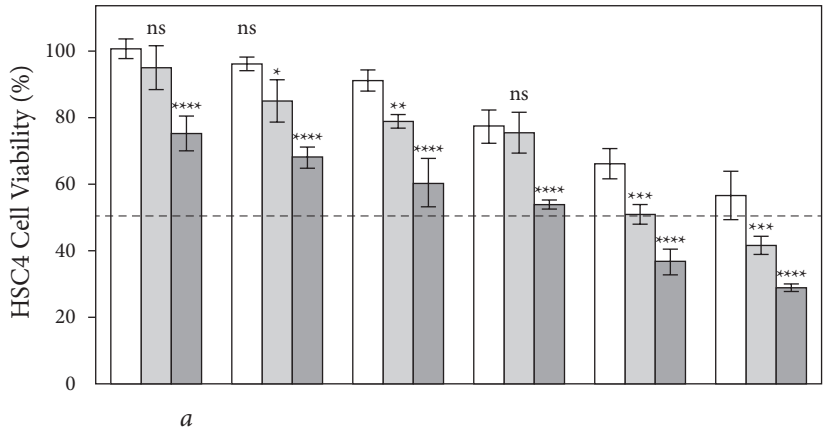
Combination of His-1 and cisplatin showed a potent synergistic inhibition of HNSCC cell proliferation. Based on the cytotoxicity data, we investigated the effect of 48 h treatment of the cells with a combination of cisplatin and His-1 in the concentration ranges lower or close to the corresponding IC_{50} values (cisplatin at concentrations lower than 1 μM, and His-1 at concentrations ranging from 2.5 to 40 μM). Our findings indicated that the combination of 0.5 μM cisplatin and 20 μM His-1 was optimal and caused the death of 50.72% HSC4 cells and 51.96% SCC25 cells (Fig. 4). The addition of 20 μM His-1 to 0.5 μM cisplatin increased the sensitivity of HSC4 and SCC25 cells to cisplatin by 27- and 14-fold, respectively. Therefore, the combined application of His-1 and cisplatin exhibited synergistic cytotoxicity.

Discussion

The considerable toxicity of chemotherapeutic drugs employed in clinical treatment poses a significant challenge for cancer therapy, particularly due to the adverse effects on normal tissues and cells [1]. Nevertheless, substances derived from diverse natural sources, known for their minimal or absent side effects, have emerged as a promising alternative or auxiliary treatment for cancer patients. Our research was focused on evaluating

HSC4-Combination

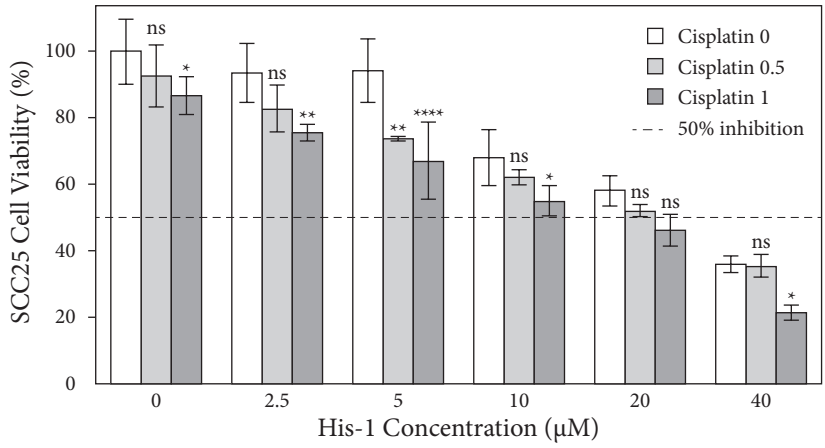
His-1 (μM)	Cisplatin (μM)		
	0	0.5	1
0	100.00	94.65	74.93
2.5	95.64	84.64	67.80
5	90.53	78.63	60.23
10	76.95	75.10	53.83
20	65.88	50.72	36.65
40	56.41	41.45	28.91



a

SCC25-Combination

His-1 (μM)	Cisplatin (μM)		
	0	0.5	1
0	100.00	92.69	86.75
2.5	93.49	82.72	75.44
5	94.16	73.53	67.09
10	68.00	62.16	54.98
20	58.13	51.96	46.09
40	35.83	35.39	21.27



b

Fig. 4. Cell viability after the combined treatment with cisplatin and His-1 for 48 h. Heat maps representation of cell viability percentage in HSC4 (*a*) and SCC25 (*b*) cells. Each experiment was performed in triplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ for each His-1 dose compared to control without cisplatin

the impact of histatin, an abundant AMP found in saliva, and cisplatin administered in combination in the HNSCC cell lines. The selection of these two molecules was based on their mechanisms of action. Cisplatin, known as an alkylating agent, induces DNA damage through the formation of cisplatin-DNA adducts, ultimately leading to the cell cycle arrest and apoptosis [1, 17]. On the other hand, His-1, due to cationic properties, binds to cancer cell membranes with increased phosphatidylserine content and causes their disruption [2, 18]. Therefore, His-1 may enhance the cytotoxic activity of anticancer drugs.

In the present study, we revealed the elevated expression levels of the *HTN1* and *HTN3* genes in SCC25 cells, whereas the HSC4 cell line predominantly expressed *HTN1* and exhibited minimal *HTN3* expression compared to normal keratinocytes. Similarly, our prior investigation unveiled higher expression levels of *HTN1* in HNSCC tumors compared to normal tissues, but no significant differences regarding *HTN3* expression were observed [19]. These discrepancies in the histatin expression may explain the varying sensitivity of different cell lines to the different species of the histatin family. It is worth noting that *HTN2*, *HTN4*, and *HTN5* are cleavage products derived from *HTN1* and *HTN3* thus not included in our study.

Despite the differential expressions of *HTN*s in these HNSCC cell lines, the treatment with synthesized His-1 peptide (representing the whole sequence of salivary *HTN1* with a phosphorylated serine residue) showed no differences in antiproliferative effects. Interestingly, our findings correspond to the study on other oral AMP, LL-37,

where the high concentrations of exogenous LL-37 significantly reduced the migration, invasion, and proliferation of the OSCC cell lines [20].

Our study provides the first evidence of the antiproliferative effect of the exogenous histatin in HNSCC cells *in vitro* that supposedly could be attributed to its cationic properties [2]. As known, AMPs elucidate their cytotoxic effects on cancer cell lines through their capacity to bind with cancer cells and kill them via direct and indirect mechanisms [8, 21—25]. A recent study demonstrated an increase in salivary AMP content including cystatin-c and His-1 in HNSCC after radiotherapy [26]. Earlier findings have underscored the differential expression of defensin and LL-37 in association with cancer development [2, 27]. In addition, research on human beta-defensin (HBD) in OSCC has revealed the downregulation of HBD-1 and HBD-2 along with the upregulation of HBD-3 [28]. Consistent with these observations, our recent results demonstrate an increased expression of *HTN1* in HNSCC compared to normal tissues [19]. Conversely, compelling evidence suggests that human LL-37 and defensins exhibit anticancer effects in various cancer types, including colon and oral cancer [21, 29—31]. Intriguingly, our study suggests that exogenous His-1 not only inhibits HNSCC cell proliferation but also synergizes with cisplatin-induced cytotoxicity, highlighting its potential as a therapeutic adjunct. His-1 sensitizes tumor cells to the cytotoxic effects of cisplatin, potentially allowing for a reduction in its effective concentration and mitigating the nonspecific toxicity associated with the treatment. Nevertheless, additional research is required to comprehensively explore the molecular mechanism of combined anticancer effects of cisplatin and histatin and assess their effects *in vivo*.

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Conflict of interest

The authors declare no conflict of interest.

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Authors' Contribution

Conceptualization, P.J, P.A; Methodology, P.J, P.A; Investigation, P.J, P.A; Formal Analysis, P.J; Validation, P.J, P.A; Data Curation, P.J, P.A; Writing — Original Draft Preparation, , P.J, P.A; Visualization, P.A. ; Supervision, P.A.; Writing — Review& Editing, P.J, P.A.

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IN VITRO АНТИПРОЛІФЕРАТИВНА АКТИВНІСТЬ ГІСТАТИНУ-1 У ПОЄДНАННІ З ЦИСПЛАТИНОМ НА ЛІНІЯХ КЛІТИН ПЛОСКОКЛІТИННОГО РАКУ ОРГАНІВ ГОЛОВИ ТА ШИЇ

Стан питання. Хіміотерапія хворих на рак органів голови та шиї асоціюється із суттєвими побічними ефектами. Антимікробні пептиди, що являють собою природні захисні молекули на кшталт дефензину-1 та LL-37, які є компонентами продуктів секреції, можуть сприяти індукції апоптозу та посиленню ефектів хіміотерапевтичних засобів. Разом з тим, такі ефекти гістатинів дотепер не досліджували. **Мета** роботи полягала у дослідженні антипроліферативної активності гістатину та його сполучення з цисплатином в лініях клітин, що походять з плоскоклітинного раку органів голови та шиї людини. **Матеріали та методи**. Експресію гена гістатину досліджували в клітинах ліній HSC4 та SCC25 методом кількісної ПЛР в реальному часі. Проліферацію клітин аналізували за різних концентрацій синтетичного пептиду гістатину (His-1), цисплатину або їх комбінації. **Результати**. Клітини SCC25 експресують гени гістатину-1 і гістатину-3, в той час як в клітинах HSC4 спостерігається лише експресія гістатину-3. Екзогенний гістатин-1 разом з цисплатином демонструє дозо-залежний синергічний антипроліферативний ефект. **Висновки**. Поєднання цисплатину в низьких дозах з гістатином є ефективним у гальмуванні проліферації клітин, що походять з плоскоклітинного раку органів голови та шиї людини. Гістатин-1 сенсибілізує пухлинні клітини до цитотоксичних ефектів цисплатину, що потенційно дозволяє зменшити ефективну концентрацію цитотоксичного хіміопрепарату.

Ключові слова: антимікробні пептиди, антипроліферативна активність, комбіноване застосування, гістатин, плоскоклітинний рак органів голови та шиї людини.