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Activity of short lytic anticancer peptides against human head and neck cancer cells *in vitro*

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Abstract

Squamous cell carcinoma of the head and neck (head and neck cancer) accounts for over 95% of all cancers of the oral cavity, oropharynx, larynx and hypopharynx [1] and it is the 6th most common cancer disease worldwide. It is the cause of 550 000 cancer deaths annually, the majority of these in the lesser developed world. [2] Treatment options are surgery, radiotherapy and chemotherapy. [1] There has been little improvement on survival the last decades, and loss of locoregional control and secondary tumors despite treatment are frequent. [1] Head and neck cancer also have been shown to have developed mechanisms to evade the immune system. [3] There is a great interest in finding new and more personalized treatment options for these patients, with targeted therapy and with immune therapy, to improve survival but also to decrease treatment related morbidity. [1], [3]. Cationic antimicrobial peptides (CAP`s) are a naturally occurring part of the innate immune system [4], and some have shown anticancer activity. [4] *De nouvo* designed shorter CAP`s have proved to kill cancer cells by cell lysis *in vitro* and by tumor lysis and concurrent immunization of the tumor *in vivo*. [5] In this study we show that two *de nuovo* designed antitumor peptides show efficacy against a panel of HN cancer cells *in vitro*, these findings indicate that treatment with lytic peptide has a therapeutic potential in head and neck cancer.

Head and neck cancer

The development of head and neck cancer is linked to alcohol consumption and cigarette smoking, and also more recently with human papilloma virus (HPV) infection. [6] HPV infected patients benefit from a paradoxally better prognosis compared to HPV negative patients. It has been suggested that this might be due to younger age and better overall health status than patients who develop head and neck cancer without HPV infection. [6] With the exception of HPV positive patients, very little progress has been reported on the disease outcome in HN cancer patients in the last 30 years. [6] Today's treatment options includes surgery, radiotherapy and conventional chemotherapy and for some patients also EGFR receptor antibodies. The prognosis for these patients is rather poor, with a 5 year survival of approximately 40-50%. [1] But despite the fact that little progression has been made to increase patient survival, it is assumed that the last decades development in surgery and organ preserving treatment regimen has had a positive impact on the patient's life quality. [1]

Background, cationic antimicrobial peptides (CAP`s)

Antimicrobial peptides are a naturally occurring part of the innate immune systems defense against prokaryote cells, fungi, protozoa and viruses, and can be found throughout the animal kingdom. [7] A large number of these natural peptides have been isolated and have been models for synthetic variants, and a number of these peptides have been reported to exhibit anticancer activity. [4] Antimicrobial peptides are a heterogeneous group of molecules, in regards to both primary and secondary structure, the majorities are short peptide(5-25aa), cationic and amphipatic. The negative charged prokaryote membrane attracts the positively charged peptides, and the amphipatic nature of the peptides enables it to interact with and disrupt lipid membranes. [4] Bovine lactoferricin is one such natural occurring CAP, from bovine milk. This peptide has been reported to exhibit antimicrobial properties, but also to kill cancer cells both *in vitro* by necrosis and apoptosis and to inhibit tumor growth *in vivo* with intratumoral injections *[8-10].* With extensive structure activity studies and optimization of the different structural parameters critical for the anticancer activity, [11-13] a family of 9-mer peptides were developed using LactoferricinB as a model peptide. These shorter peptides have a α –helical secondary structure and contains one bulky unnatural (non coded) amino acid. They effectively kill cancer cells *in vitro,* even cell lines known to be resistant to conventional cytostatic drugs. *In vivo* they cause tumor regression after intratumoral injections, and in immune competent mice causes long term protection even when reinocculated, apparently immunizing the animal against the tumor. [5]

Methods

Peptides

LM1 peptide was synthesized in the peptide laboratory at UiT by the author, using solid phase method using Fmoc chemistry on a Pioneer Peptide synthesizer (Applied Biosystem, Foster City, CA) and purified using high-pressure liquid chromatography (HPLC, Waters Corporation, Milford, MA, USA). The integrity of the peptide was confirmed my mass spectrometry (VG instruments Inc.) LM2 peptide was made to order by Bachem.

Both peptides were 9-mers, containing one unnaturally occurring, bulky amino residue. The main difference between these two peptides is the placement of this residue.

Cell lines

Primary squamous cell carcinomas cells from human head and neck cancer patients were obtained from cancer patients as previously described [14]. Cells were cultured in high glucose DMEM medium containing 1% L-glutamine, 10% fetal bovine serum. Cell lines were purchased from ATCC, (Manassas, VA). FEMX, HT-29, MDA-MB-435 were cultured in RMPI containing 1% L-glutamine and 10% fetal bovine serum, DU-145, ATCC-SCC 9 and 25 were cultured in high glucose DMEM containing 1% L-glutamine and 10% fetal serum albumin. All cell cultures were maintained in absence of antibiotics in a humidified atmosphere at 37C and 5%CO2.

Viability assay, MTT

This assay is used to determine the amount of viable cells after treatment with cytotoxic reagents, and the method is widely used in drug development studies . It is a fast, reliable and cost effective way to determine toxic effect of drugs and cell viability. MTT is added to cells after the desired treatment, and will penetrate into cells and in the mitochondria be reduced to a formazan product that can be dissolved in the media after alcohol lysis of the cells. The absorbance can be measured by a plate reader at 590nm, and the amount of formazan in each well is directly proportional with the amount of living cells. The percentage of living cells is calculated by using the following equation: (A=absorbance)

(A treated cells – A positive control)/ (A negative control – A positive control) X 100

Cells were cultured until a confluence of 80%, trypsinated and a sample of the cells were counted. Cells were transferred to a falcon tube, centrifuged at 3000rpm for 5 minutes before they were diluted in growth medium and seeded into 96 wells plate. Number of cells depending on cell line, 10 000, 15 00 or 20 000 cells per well in 100 uL growth medium. Cells were then allowed to attach for 16 hrs. Cells were washed with serum free RPMI (assay medium) twice before treated with peptide. Peptide was weighed at 1mg, then dissolved in assay medium to a concentration of 2000ug/ml, and thereafter diluted in 8 concentrations from 10-500ug/ml, and added to the cells with 3 parallels for each concentration. Positive control was 1% Triton-X and negative control was assay medium only. Cells were incubated with peptide for 4 hours before 10 uL MTT was added to each well followed by

2 hours incubation. Then 70 uL media was carefully removed from each well before 100uL acid isopropanol was added. The plate was gently mixed before absorbance was measured. Final results were calculated using the mean of three experiments, each with triplicate wells. For a few of the cells, the experiment was only conducted twice due to extreme slow growth. (UT-SCC 16, 33 & 48)

Results

Both peptides are effective against HN cancer cell in vitro

The efficacy of the short lytic lytic peptides against HN cancer cells was determined with MTT-assay and IC-50 value was compared to the IC-50 value of 4 human cancer cell lines known to be sensitive to peptide treatment. (Table 1 & 2, figure 1 & 2) Median IC-50 value for LM1 peptide was 58,5 uM in the HN cancer cells and 56,5 uM in the other cancer cell lines tested. For LM2 peptide the median value was 26,5 uM in the HN cancer cells and for the other cancer cells the median value was 25 uM. Table 1 and 2 and figure 1 & 2. It should also be noted that this effect was measured after a short incubation time, of only 4 hours.

Table 1

LM-1 peptide

HN SCC cells	IC-50 µM
UT-SCC-16A	31
UT-SCC-59C	38
ATCC-SCC-9	43
UT-SCC-34A	43
UT-SCC-24A	48
UT-SCC-1A	52
UT-SCC-48 *	65
ATCC-SCC-25	69
UT-SCC-33	76
UT-SCC-63A	82
UT-SCC-54B	100
UT-SCC-9	109

Other cancer cells	IC-50 µM
MDA-MB-435 Melanoma	39
FEMX Melanoma	53
HT-29 Colon carcinoma	60
DU-145 Prostata	69

Normal cells	IC-50 µM
MRC-5 human fibroblast	129

Table 2

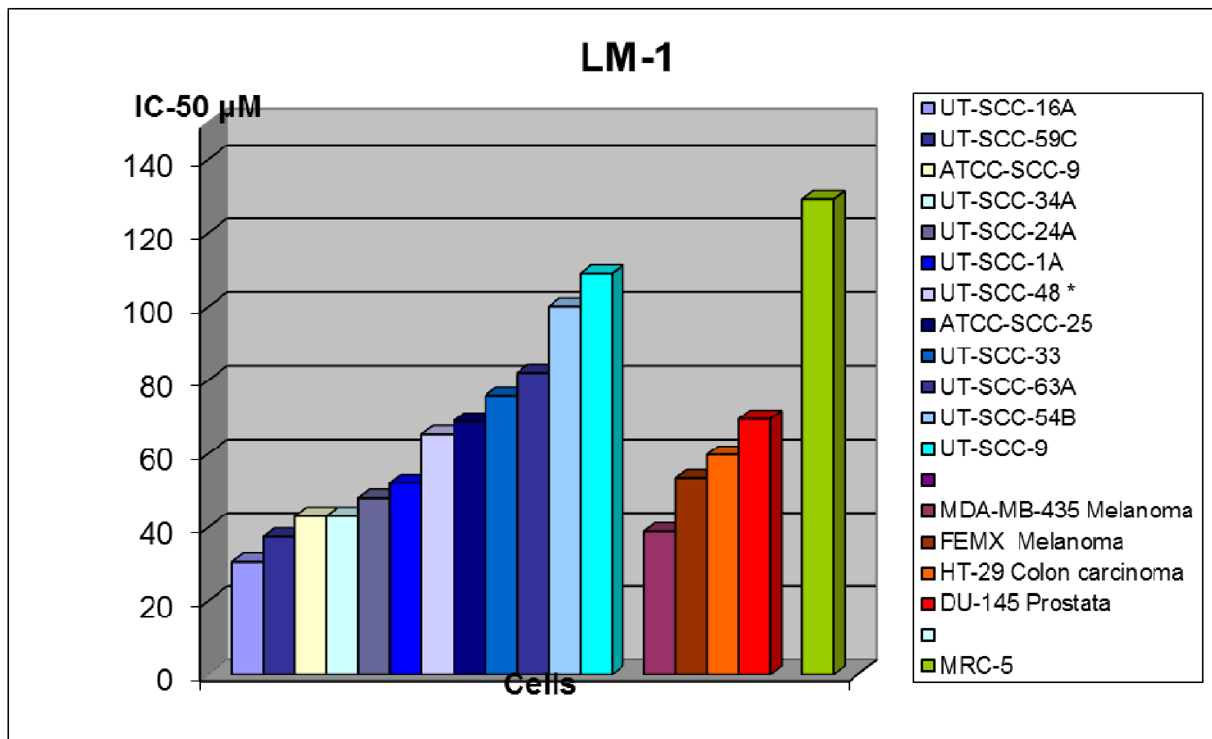
LM-2 peptide

HN SCC cells	IC-50 µM
UT-SCC-16A *	15
UT-SCC-59C	17
UT-SCC-34A	17
UT-SCC-24A	17
ATCC-SCC-9	22
ATCC-SCC-25	26
UT-SCC-1A	27
UT-SCC-63A	29
UT-SCC-33 *	30
UT-SCC-48 *	32
UT-SCC-54B	34
UT-SCC-9	38

Other cancer cells	IC-50 µM
MDA-MB-435 Melanoma	17
FEMX Melanoma	24
DU-145 Prostata	26
HT-29 Colon carcinoma	34

Normal cells	IC-50 µM
MRC-5 human fibroblast	26
HUVEC human endothel	23

*IC-50 Values of the LM-1 peptide (Table 1) and the LM-2 peptide (Table 2). *tested only twice.*



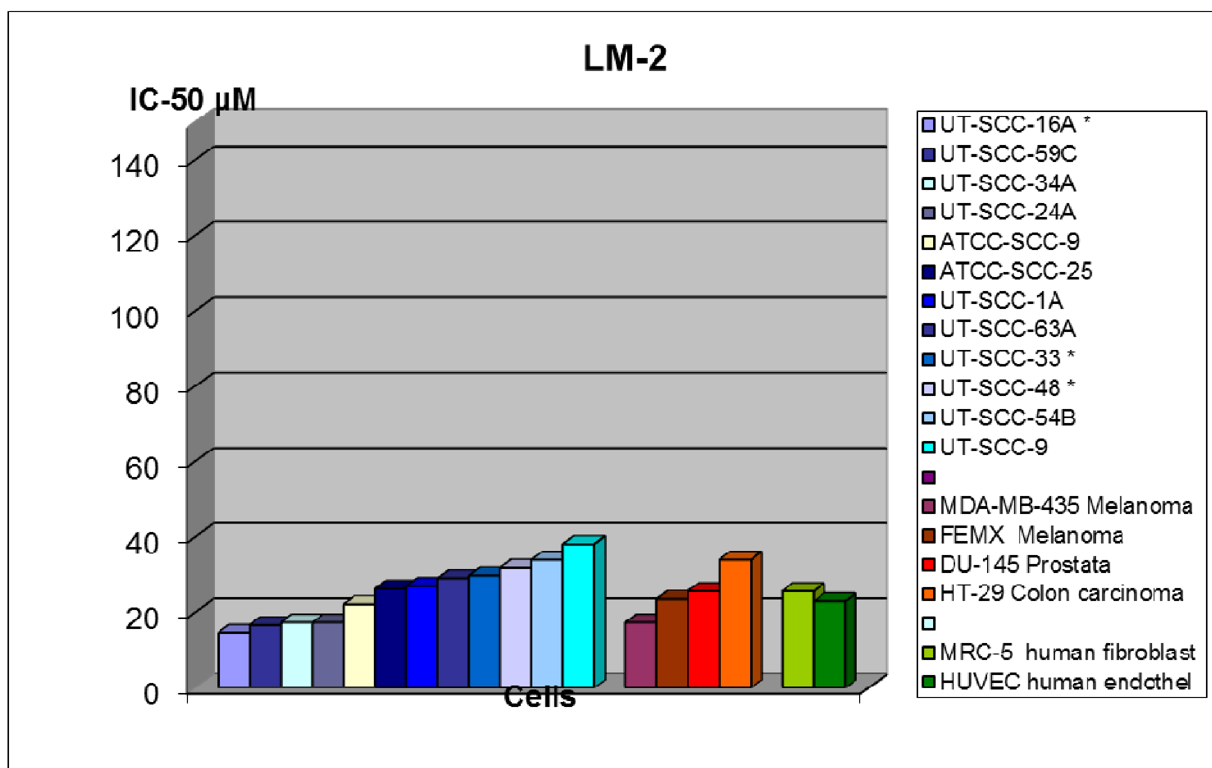


Figure 1 & Figure 2: IC-50 values of the tested LM-1 and LM-2 peptides. Blue colored colonna's represent HN cancer cells, red colored represent other cancer cell lines and green represent untransformed cells.

Differences in peptide sensitivity between cells

The HN cancer cells tested showed diversity in sensitivity for both the peptides tested. (Table 1 & 2, figure 1 & 2) The differences were most pronounced for the LM-1 peptide was the sensitivity ranged from 31uM in the most sensitive culture, to 109uM in the most resistant culture. For the LM-2 peptide HN cancer cells demonstrated IC-50 values ranging from 15 to 38uM. It was much the same pattern of sensitivity for both peptides. There were also a varying sensitivity for the peptides amongst the other cancer cell lines tested, but none showed as high IC-50 values as the most resistant HN cancer cells. Also for the other cancer cells, largely the same pattern of sensitivity could be recognized between the two different peptides tested.

Differences in activity between LM-1 and LM-2 peptide

For the HN cancer cells, the LM-1 peptide had a mean IC-50 value of 63uM while LM-2 had a mean IC-50 value of 25,3uM. For the other cancer cells the mean IC-50 value for the LM-1 peptide was 55,3uM and for LM-2 peptide the mean IC-50 value was 25,3 uM. The LM-2 peptide showed greater activity compared to the LM-1 peptide, and also a more homogenous activity between the different cultures. (Table 1 & 2, figure 1 & 2)

Discussion

The peptides tested showed diverse between the cell lines, and also between the different peptides. Both peptides showed activity against the human HN cancer cells, as compared to the activity against the four cancer cell lines that were tested. These data indicates that lytic peptides may be a novel therapeutic approach against HN cancer in human patients. Also it can be assumed that the characteristics that is suggested to make cancer cells more sensible for peptide treatment, namely increased membrane fluidity [15], increased membrane surface due to a high amount of microvili [16] and the relative amount of different charged membrane components like phosphatidylserine [17], are also present in the squamous cell carcinoma cells of the head and neck cancers, as in many other cancer cells.

The two tested peptides also shows a difference in specificity against cancer cells compared to normal cells. While the LM1 peptide showed a greater activity against cancer cells as compared to normal human fibroblasts, the LM-2 peptide were also active against normal human fibroblasts and endothelial cells. When considering the suggested intratumoral administration of the peptide treatment in human patients, this lack of specificity toward tumor cells might not be a disadvantage. It is known that cells in the tumor microenvironment are contributing to the tumor progress and that this tumor-host interaction is crucial for the tumors growth, ability to metastasize and might even contribute to drug resistance [18]. Thus targeting the whole tumor mass included associated vessels and fibroblasts might be more effective than lysis of the tumor cells alone. Considering the patient safety, the LM-2 peptide has previously shown no hemolytic activity, and also the half-life in plasma is approximately 15 minutes (data not shown).

The variety of the sensitivity between different cells is notable, especially in regard to the LM-1 peptide. The most resistant cell culture had an IC-50 value more than a threefold over the most sensitive cell culture. This might be caused by different membrane composition in the more resistant

cells; a less negatively charged membrane surface would be less likely to attract the cationic peptides to its surface. A higher amount of cholesterol would likely help stabilize the membrane and inhibit membrane collapse at a lower concentration, thus elevating the IC-50 value. These are two examples of how a different membrane composition might inhibit the peptides killing effect on some cell types. It has also been proved that the LM2 peptide in lower concentrations enters the cells cytoplasm and causes destabilization of the mitochondrial membrane (data not shown, authors "Forskerlinjeoppgave"), and it is likely that the effect of this mitochondrial attack contributes to the cytotoxicity in lower concentrations. It is shown that cancer cells might also harbor changes in the mitochondria like hyperpolarization [19] and it is not unlikely that this mitochondrial transformation might render the cell more susceptible for peptide attack. The number of mitochondria also varies greatly amongst different cell types, and this might also influence the sensitivity of a certain cell line to the peptide treatment. It is also known that HN cancers also harbors a great heterogeneity anatomically, even to such a degree that it might be discussed whether they might be considered one disease entity. [20] Different anatomical sites means different microstructure, different microenvironment, including blood supply, venous and lymphatic drainage. All this would quite possibly lead to different phenotypes, and could influence on the sensitivity of the cell lines to peptide treatment.

Conclusion

This panel study demonstrates that the short lytic anticancer peptides LM1 and LM2 are effective against human head and neck cancer cell lines, with the LM2 peptide being the most active. The tested cell lines also shows a variety in sensitivity, this result is as expected considering the multiple anatomical locations the tested cell lines were derived from.

Further studies *in vitro* should include testing against cells from so called pre-malignant lesions (leukoplakia and eryplakia) which may prelude malignant transformation in the squamous epithelia of the oral cavity. Also further studies should aim at testing the peptide on HN cancer cells in experimental animal models.

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