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Development of a cell penetrating peptide carrying a biological active HPV16 E6 inhibitory molecule for delivery into cervical cancer cells

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Introduction and Aim

The E6 oncoprotein encoded by high-risk human papillomaviruses (HPV) plays a crucial role in the development of cervical cancer mainly via degradation of the tumor suppressor protein p53. HPV E6 protein forms an heterodimer with the E3 ubiquitin ligase E6 associated protein (E6AP), which recruits p53 causing its ubiquitination and proteasomal degradation. Yeast two-hybrid screening of a randomized peptide expression library previously allowed the identification of the E6-binding 15-mer peptide, named pep11, which inhibited the formation of the E6/E6AP complex. The intracellular expression of pep11 caused the specific inhibition of colony formation of HPV16-positive cancer cells, abrogated p53 degradation and induced cell death selectively in HPV16-positive cancer cells[(1)]. Several attempts were made to chemically synthesize pep11, however, due to its aggregation tendency and high hydrophobicity, the yields were very poor and the peptide was insoluble in aqueous solution. Then, subsequent studies focused on the development of a modified pep11 sequence named pep11**[(2)].

We aimed to chemically synthesize a modified pep11 (CPP-pep11) containing a short cell penetrating peptide (CPP) moiety bound to the N-terminal end of the original pep11, in order to facilitate its solubility and delivery into HPV-positive cancer cells.

CPP - pep11

Biot-RRRRRR-EFGSGCSCIVCIGLI-NH_2

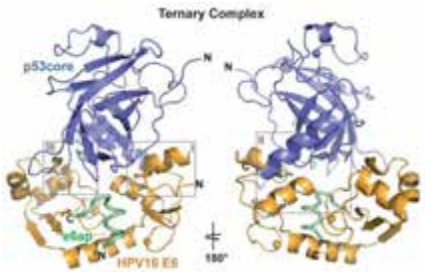

Figure 1: Structure of the HPV16 E6/e6ap/p53core ternary complex (green: E6AP peptide, gold: HPV16 E6, purple: p53 core[(3)]. Biotin was bound to the N-terminus.

Methods

Boc solid-phase peptide synthesis: The RRRRRR-EFGSGCSCIVCIGLI-NH2 (CPP-pep11) peptide was synthesized, at Laboratory of Biochemistry and Molecular Biology (Institute of Human Virology, Baltimore), on an automated peptide synthesizer, Applied Biosystems ABI 433, adopting the 0.250 mmol scale and using N,N-Diisopropylethylamine (DIPEA) in situ neutralization/HBTU activation protocol for Boc solid-phase peptide synthesis. Biotin at N-terminus was conjugated manually adding 1.2 equivalents of Biotin N-hydroxysuccinimide ester dissolved in DMF containing 2 equivalents of DIPEA. The coupling time was overnight at room temperature under stirring. The peptides derivatives were treated for the cleavage with anhydrous hydrofluoric acid (HF) in the presence of 5% p-cresol at 0 °C for 1h, followed by precipitation with cold diethyl ether. The precipitates were dried and solubilized in water (0.1% TFA) for the purification.

Analysis and purification: The crude compounds were purified by reverse-phase high-performance liquid chromatography RP-HPLC using a Waters C18 column eluted with H2O/0.1% TFA (A) and CH3CN/0.1% TFA (B) with linear gradient from 20 to 40% B over 2h at 40ml/min. Molecular weights were verified by electrospray ionization mass spectrometry (ESI-MS).

Cell culture and CPP-pep11 treatments: HPV16 positive cervical cancer-drived SiHa cell line (p53 wild type) were used for these studies. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) high glucose supplemented with 10% fetal bovine serum (FBS), 2mM L-Glutammine and 100U/ml Penicillin - 100µg/mL Streptomycin at 37°C and 5% CO2. Cells were seeded in 6-well plate (3 x 10^5/well) and treated at decreasing concentrations of 20, 10, 5, 2, 1 µM for 5h, 24 and 48 h to monitor CPP stability at longer time and alteration in p53 levels.

Western blotting analysis: Cells treated with CPP-pep11 were washed three times with PBS1x and scraped into 300 µL RIPA buffer. Protein concentration was determined by Bradford method and 20 µg of proteins separated by electrophoresis either in 10% SDS-polyacrilammide gel, to detect cell proteins, or in 16% Tricine-SDS-page gel to detect CPP-pep11.

Confocal microscopy: SiHa cells were grown on glass coverslips in 6/well plate at a density of 3 x 10^5 and incubated for 24 hours in DMEM 10% FBS and then treated with CPP-pep11 and CPP-Scrpep11 20 µM, 2 µM and 0,5 µM for 5 h. Cells were fixed in 4% formaldehyde for 10 min at RT and permeabilized with 0.1% Triton ×100. The CPP-pep11 conjugates were incubated for 2 h. After incubation, the membrane-bound cargo was quenched with avidin. The intracellular cargo was then visualized with fluorescent streptavidin (streptavidin-Alexa488, in green). Nuclei were stained with DAPI (in blue), Phalloidin in red, CPP in green, DAPI in blue for nuclei.

Results

We have synthesized via Boc chemistry an optimized pep11 peptide containing a short cell penetrating peptide (CPP) at the N-terminus end (CPP-pep11). The CPP-pep11 was successfully synthesized and was further reacted with biotin. The CPP-pep11 yielded a 95-97% pure peptide, which was soluble in water at 20 µM concentration. The ability of CPP-pep11 to spontaneous penetrate cell membrane was evaluated on HPV-positive cervical cancer SiHa cells, which were incubated with serial dilutions (10, 5, 2, 1 µM) of biotin-labelled CPP-pep11 (bio-CPP-pep11) for 24, 48 and 72 hours. The cell uptake and intracellular distribution was evaluated by confocal microscopy (Figure 2. A-B). The CPP-pep11 at 2uM concentration showed an optimal internalization efficiency with homogenous distribution in the cytoplasm and in the nucleus. However, 20uM concentration of CPP-pep11 affected cell viability causing rupture of cell membranes. Western blot analysis of biotinylated CPP-pep11 in the cells showed levels of peptide concordant with the input, demonstrating stability over the time of CPP-pep11 in the cells.

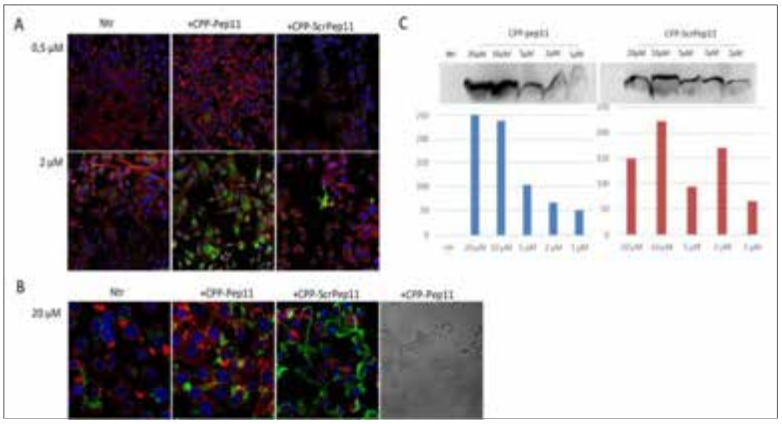

Figure 2: (A, B) Immunofluorescence of SiHa cells after treatment with CPP-pep11. The biotinylated internalized CPP-pep11 was visualized by confocal microscopy.
(C) immunoblotting of SiHa cellular exctracts afte 5 hours treatment with 20, 10, 5, 2 and 1 µM of CPPs. Lower, the graph indicates the CPP amount in the cells compared to the concentration used. The uptake of CPP-pep11 reduces on decreasing of micromolarity; for CPP-Scrpep11 is variable.

Conclusions

In conclusion, the addition of a CPP sequence to pep11 allowed to obtain a good yield of an extremely hydrophobic pepted, to enhance its solubility and to facilitate the uptake into the cells- The CPP-pep11 might be a promising anti-cancer moiety. Indeed, the CPP-pep11, by restoring the tumor suppressor function of p53, may hold promise as a potential therapeutic strategy for treating HPV-positive cervical carcinoma.

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