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# Intracellular Delivery of Histidine and Arginine Rich Cell Penetrating Peptides into HeLa cell Line

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## ABSTRACT

Most bioactive macromolecules, such as DNA and RNA, cannot permeate into cells freely from outside the plasma membrane. Cell penetrating peptides are a group of short peptide sequences that are able to transverse the cell membrane for mediating gene into living cells. In this study we demonstrate two cell penetrating peptides 5H-R9-C and 7H-R9-7H-C. These peptides are synthesized by solid phase methodology and labelled these peptides by FITC and purified them by RP-HPLC. FITC labelled peptides are efficiently internalize into HeLa cells confirmed by fluorescence microscopy and flow cytometry. Further studies are being carried out to deliver cargo molecule (siRNA, oligonucleotide and pDS RED) by these peptides. These results suggest that these peptides appear to be a promising tool for drug delivery.

**Keywords:** Fluorescein isothiocyanate (FITC), Reverse phase high performance liquid chromatography (RP-HPLC), Small interfering RNA (siRNA)

The internalization of exogenous materials through plasma membranes by endocytosis is an important function of eukaryotic cells (Mukherjee *et al.* 1997). Cell-penetrating peptides (CPPs), also known as protein transduction domains (PTDs) or arginine-rich intracellular delivery (AID) peptides, are a group of short peptides with the ability to penetrate cell membrane, thus have been considered as candidates for mediating drug delivery (Stewart *et al.* 2008). CPPs are amphipathic, hydrophobic or cationic peptides (Wagstff *et al.* 2006). Transactivator of transcription (TAT) and penetratin derived amino acids 48-60 of HIV-1 and Drosophila melanogaster homeodomain protein Antennapedia (Antp) respectively are typical examples of protein-derived CPPs (Zorko *et al.* 2005). The kinetics of cellular internalization varies with the different CPPs (Eiricksdocttir *et al.* 2010). CPPs are powerful penetrating biomaterials that are able to deliver bioactive macromolecules (cargoes) such as proteins, nucleic acids, peptide nucleic acids, inorganic particles and liposomes into cells of various species (Chang *et al.* 2007; Liu *et al.* 2008; Chen *et al.* 2007; Wang *et al.* 2007). In a previous study internalization of CY3 labelled plasmid DNA into the insect cells with non covalent strategy was demonstrated by arginine and histidine rich peptide HR9 (Chen *et al.* 2012). Several studies have found that

arginine-rich CPPs, such as nona-arginine (R9) peptide, destabilized the plasma membrane creating transient pores for cellular penetration (Herce *et al.* 2009). Lo and Wang discovered that a PTD of TAT comprised of additional polyhistidine and cysteine residues possessed endosomolytic properties and increased the gene expression of DNAs carried by this CPP ( Lo *et al.* 2008). In the present study we have synthesized histidine and arginine rich CPPs; 5H-R9-C and 7H-R9-7H-C peptides by solid phase peptide synthesis, labelled these peptides with FITC and purified all these peptides with RP-HPLC, and characterized them for their internalization in HeLa cells by fluorescence microscopy.

## MATERIALS AND METHODS

### Peptide synthesis

Solid phase peptide synthesis with Fmoc chemistry was used to carry out synthesis of peptides 5H-R9-5H-C and 7H-R9-7H-C. In brief, after 2 h swelling of Wang resin (loading capacity 0.90 mol/g of resin, Nova Biochem), first amino acid i.e. Fmoc–Cys (Trt)–OH (five equivalent to the loading capacity of the resin) was added along with equivalent amounts of DIPC and DMAP. Coupling was allowed for 2 h followed by end capping with acetic anhydride. Loading/coupling efficiency was determined by estimating Fmoc group at each step of synthesis. Using 20% piperidine (v/v), Fmoc group from Cys-linked resin was removed. The next amino acid was histidine (3 equivalents) activated using 2-(1H-benzotriazolyl-1-yl)-1,1,3,3-tetramethyuroium-hexafluoro phosphate and 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid (HBTU-HOBT), was made to react with Cys on the resin support. Coupling efficiency was measured, and then, next amino acids were added by the similar steps of synthesis. Subsequent deprotection, coupling and end capping were repeated till the completion of synthesis. Peptide was deprotected and cleaved from resin beads using a cleavage mixture composed of Trifluroaceticacid/phenol/thioanisol/1,2-ethanedithiol/water (82.5:5:5:2.5:5 v/v) for 4 h and precipitated in chilled dry diethyl ether.

### RP-HPLC purification

Precipitated peptides in crude form were purified by reversed-phase chromatograph (RP-HPLC) on a C18 semi-preparative column (300 × 7.8 mm; 5.2 µ particle size) using RP-HPLC pump system (Shimadzu, Tokyo, Japan) fitted with a photo diode array (PDA) detector. Binary gradient of water/acetonitrile having 0.1 % TFA (v/v) was used for purification of peptide.

### FITC labelling of the peptides

1.1equimolar quantity of FITC dye was reacted with bead bound peptides overnight at room temperature. After thorough washing, beads were dried in methanol and peptides were cleaved from beads and precipitated in diethyl ether. Finally, cleaved peptides were purified using RP-HPLC.

### Cell culture

HeLa cells were maintained in DMEM supplemented (Hyclone) with 10% fetal bovine serum and 1X penicillin-streptomycin (Gibco). Cells were washed with PBS three times before and after each treatment.

### Transfection of peptide to cells

HeLa cells were overlaid in a 24 well plate ($2 \times 10^6$ cells/well) in 10% Growth medium (DMEM and 10% FBS supplemented with penicillin-streptomycin).

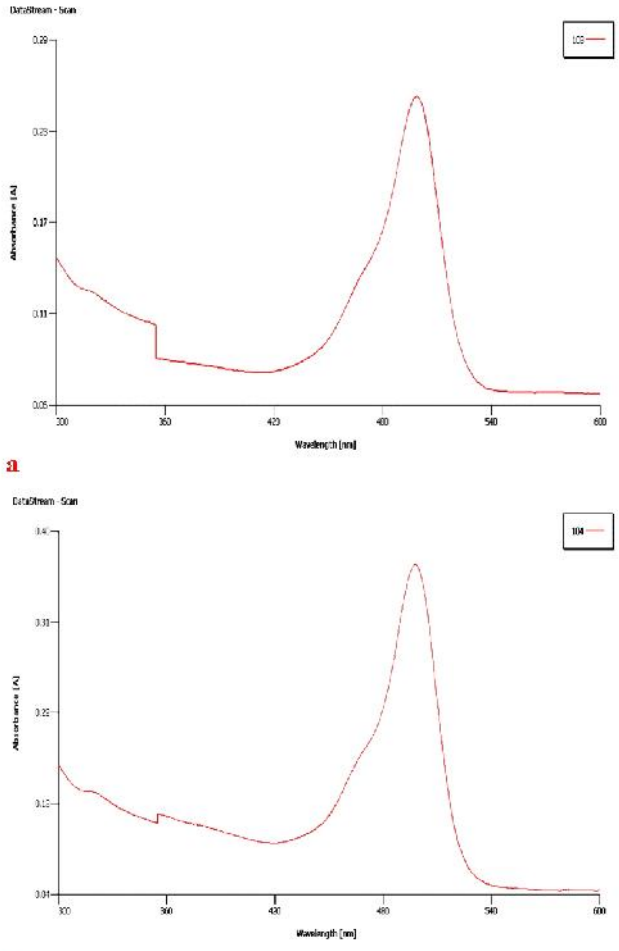


**Fig. 1 (a-b):-** Absorption spectra of FITC labelled peptides 5H-R9-5H-C and 7H-R9-7H-C respectively. The peak at 490 nm suggest that peptide is FITC labelled. Peptides were purified byRP-HPLC (Fig. 1a and 1b). Both

**Table 1. List of arginine and histidine rich CPP**

| Sl. No. | Peptide Sequences | Mol. Weight | PI | Net charge at pH 7.0 |
|---|---|---|---|---|
| 1 | HHHHHRRRRRRRRRC | 2212.56 g/mol | 12.94 | 9.4 |
| 2 | HHHHHHHRRRRRRRRRR HHHHHHHC | 3446.83 g/mol | 12.94 | 10.2 |

FITC labelled peptides were transfected into HeLa cells (1µg) in DMEM for 1 h at 37$^{O}$C for their transfection efficiency.

Transfection was done with FITC labeled peptide at 60-70% cell confluency. For this different amounts of 5H-R9-5H-C and 7H-R9-7H-C FITC labeled peptide (0.5µg and 1µg) were incubated with HeLa cells for 1 h in serum free DMEM.

After transfection cells were washed with PBS (phosphate buffer saline) by three times and cells were fixed in absolute alcohol for 20 minutes, again washed the cells with PBS and cells were stained with DAPI for nuclear localization. The cells were observed under fluorescence microscopy. In addition, flow cytometry was done to quantitate internalization. The cells were transfected with 1µg of both the peptides for 1h at 37$^{0}$C and after transfection cells were trypsinized using 0.25% trypsin, centrifuged at 3000 rpm and washed with PBS two times and analysed for flow cytometry under FL1 filter, the untransfected cells taking as a negative control.

## RESULTS

In this study we synthesized two histidine and arginine rich peptides H5-R9-C and H7-R9-H7-C (Table 1) by solid phase methodology. These peptides were labeled with FITC and confirmed spectrophotometrically by scanning peptides from 300-600 nm, λmax was observed between 420-500nm. FITC labeling was also confirmed by recording fluorescence spectra at 490 nm excitation which gave an emission maximum at 520 nm (Fig. 1 a&b).

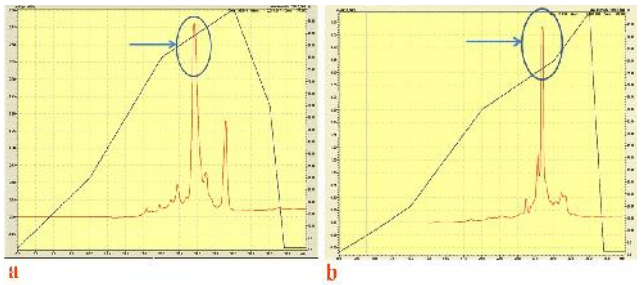


**Fig. 2 (a-b) :-** RP-HPLC chromatogram of 5H-R9-5H-C and 7H-R9-7H peptides respectively on a semi-preparative column (Phenomenex, Luna, C18, 300 × 7.8 nm 5.2µ size), tented line (black) indicate the gradient profile of solution B (ACN with 0.1% TFA, v/v in solvent A (water with 0.1 % TFA, v/v).

No green fluorescence was observed in the cells treated with PBS as a negative control using a fluorescence microscope (Nikon) (Fig. 3g). On the contrary green fluorescence was visualized in the cells treated with both the FITC labelled peptides (Fig. 3a & 3d). Colocalization study by merging images of blue and green channels revealed subcellular localization of the peptides in both cytoplasm and nucleolus (Fig. 3c and 3f). For the quantitation of the internalization the flow cytometry was performed, The transfected cells were trypsinized with 0.25% trypsin and analysed under FL1 filter. Untrasfected cells were taken as a nagative~~positive~~ control. It was observed that 89% cells were found to be positive by H7-R9-H7-C (Fig 4a) peptide and 93% cells are found to be positive by the transfection of H5-R9-C peptide (Fig 4b). These results confirmed cell penetrating ability of H5-R9-C and H7-R9-H7-C peptide.

The MTT assay was also performed to observe the cell cytotoxicity, and it was found that the peptide was not cytotoxic even at high concentration (data not shown).

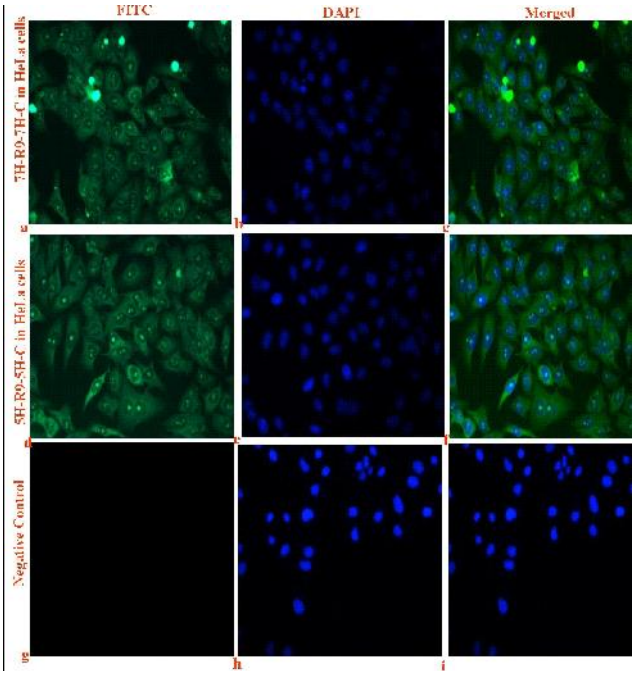


**Fig. 3 (a-i):-** Fluorescence micrograph showing internalization of 5H-R9-5H-C and 7H-R9-7H-C peptide in HeLa cells; a:- Internalization of FITC labelled 7H-R9-7H-C peptide (1µg) d:- Internalization of FITC

labelled 5H-R9-5H-C peptide (1µg) g:- Negative control cells in PBS; (b,e & h):- Nuclear staning with DAPI in respective cells; (c, f & i):- Superimposition of green and blue (merged image).

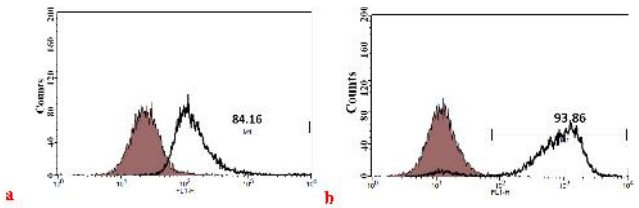


**Fig. 4(a-b):** Flow cytometry overlay histograms showing internalization of FITC labelled peptides in HeLa cells, (a) Internalization of FITC labelled H14R9C peptide in HeLa cells. (b) FITC labelled H5R9C peptide. The First graph is the control cells (grey colour) and overlay graph (Black line) showed the internalized positive cells.

## DISCUSSION

In recent years, CPPs were efficient and safe tools for gene transport. In this report we demonstrate arginine and histidine rich CPPs (H5-R9-C and H7-R9-H7-C) that can form a stable complex with a fluorophore (FITC) and can be efficiently delivered into cancerous cell lines. We observed that H5-R9-C peptide is very much efficiently delivered into the cells. By flow cytometry quantitation it is clearly observed~~shown~~ that the H5-R9-C peptide more efficiently internalized into cells. The guanidinium residues of arginine are the essential ingredients of a peptide's ability to enter cells has allowed for the design of a range of guanidinium-rich synthetic analogs. Moreover the peptide length should be 15-20 aa long. Thus, this peptide appears to be the most excellent carrier among arginine-rich CPPs tested here for delivery in HeLa cells. In general, nonviral delivery systems including cationic liposomes and synthetic polymers have been developed as a means for gene delivery (Luo *et al*. 2000). Recently, CPPs have gained broadening popularity as promising non-viral delivery vectors. For coupling of nucleic acids to CPPs, two distinct strategies may be applied—covalent and noncovalent (Margus *et al*. 2012 The noncovalent strategy has been developed for the delivery of peptides, proteins, nucleic acids, oligonucleotides, and nanoparticles. To develop clinical applications of CPPs, many criteria have to be met, such as effective transfection activity, bio-distribution, safety, and manufacturing (Margus *et al*. 2012). The non-viral DNA delivery mediated by CPPs provides advantages overexisting methodologies for gene therapy, such as its ease of assembly, high transfection efficiency, broad cargo capacity, and low levels of adverse effects of CPP-mediated delivery, In terms of biosafety, nonviral systems are relatively safer than viral delivery systems, but they often suffer from an unsatisfactory transfection efficiency (Nakase *et al.* 2010). CPPs possess the ability to not only efficiently internalize but also deliver cargos into various types of live cells (Dowdy and Snyder, 2005). Lo and Wang designed a C-5H-TAT-5H-C peptide, consisting of a PTD of TAT flanked by 5-histidine residues and a cysteine at both ends (Lo *et al*. 2008). This peptide promoted internalization of a nonviral DNA vector and increased its gene expression. They proposed that the imidazole groups of histidine facilitated proton in flux (proton sponge effect) to endosomes, leading to endosomal bursting (Lo *et al*. 2008). Thus, it should be note that membrane transduction involves multiple factors, such as degree of hydrophobicity, peptide structural transitions and membrane composition (Ziegler *et al*. 2008). Studies have shown that the guanidinium-head groups of arginines were crucial for cellular uptake due to their interactions with membrane phospholipids, as substituting nitrogen of guanidine with oxygen reduced the ability of transduction (Ziegler *et al.* 2008, Wright *et al.* 2003). It supports this notion that the penetration rate of the guanidinium-rich peptides was about three times faster than that of the PTD of TAT (Wender *et al.* 2000). The hypothesis was supported by our findings that the H5-R9-C and H7-R9-H7-C peptides are more efficiently delivered into the HeLa cells with no cytotoxicity. In future, the challenges associated with CPP-mediated delivery of nucleic acids in vivo include systemic concerns (packaging, toxicity, immunogenicity, serum stability, blood clearance, and target selectivity) and intracellular trafficking (endosomal release).

## CONCLUSION

Two novel CPPs, H5R9 and H14R9, were synthesized by solid phase peptide synthesis using Fmoc chemistry and labelled with FITC to assess the internalization ability in HeLa cell line. These peptides have the ability to go inside the HeLa cells as evident by fluorescence microscopy and flow cytometry. Electrostatic interactions play a key role in cellular internalization. Further studies are being carried out in our laboratory for the delivery of cargo such as oligonucleotide, plasmids and siRNA using these peptides.

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