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Research Article

The Relation Between Thermodynamic and Structural Properties and Cellular Uptake of Peptides Containing Tryptophan and Arginine

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Abstract

Purpose: Cell-penetrating peptides (CPPs) are used for delivering drugs and other macromolecular cargo into living cells. In this paper, we investigated the relationship between the structural/physicochemical properties of four new synthetic peptides containing arginine-tryptophan in terms of their cell membrane penetration efficiency.

Methods: The peptides were prepared using solid phase synthesis procedure using FMOC protected amino acids. Fluorescence-activated cell sorting and fluorescence imaging were used to evaluate uptake efficiency. Prediction of the peptide secondary structure and estimation of physicochemical properties was performed using the GOR V method and MPEx 3.2 software (Wimley-White scale, helical wheel projection and total hydrophobic moment).

Results: Our data showed that the uptake efficiency of peptides with two tryptophans at the C- and N-terminus were significantly higher (about 4-fold) than that of peptides containing three tryptophans at both ends. The distribution of arginine at both ends also increased the uptake efficiency 2.52- and 7.18-fold, compared with arginine distribution at the middle of peptides.

Conclusion: According to the obtained results the value of transfer free energies of peptides from the aqueous phase to membrane bilayer could be a good predictor for the cellular uptake efficiency of CPPs.

Introduction

Transfer of cargos across cellular membranes is a major issue in cell biology and medicine. During the past few decades, significant progress has been made in focusing on the manufacture of new carriers for cargo delivery into cells. Cell-penetrating peptides or protein transduction domains (PTDs)[1] are new carriers that have been widely used to promote the uptake of various macromolecules. CPPs have been shown to efficiently improve transportation of PMO (phosphorodiamidate morpholinos),[2] PNA (peptide nucleic acids),[3] DNA,[4] siRNA (short interfering RNA),[5] drug,[6] and nanoparticles[7] across biological membranes. Recently several classes of CPPs have been introduced. Model amphipathic peptides (MAP) are one of the most common classes of CPPs that have been used in scientific research.[8] Amphipathic peptides contain hydrophobic and cationic residues in the backbone, this class of peptides have the ability to enter cells and can have antimicrobial properties.[9]

A group of amphipathic peptides containing tryptophan and arginine residue have been widely used as CPP for delivery of various drug and biological compounds.[10-12] Tryptophan has an aromatic side chain[13] and can participate in hydrophobic interactions to the lipid hydrocarbon chains of the cell membrane.[14] Arginine is a hydrophilic and basic amino acid;[15] the guanidino groups of arginine tend to bind with phosphate in the cell membrane lipids.[16] In this study, four amphipathic peptides containing tryptophan and arginine (RRWWWWRR, RRRWWWWRRR, WWRRRRWW and WWWRRRRWWW) were designed and synthesized in order to explore the relationships between the structural/physicochemical properties and penetration into the MCF-7 cells.

Materials and Methods

Materials

Fmoc–Trp (Boc) -OH, Rink amid AM and Fmoc-Arg (Pbf) -OH were purchased from AAPPTec (Louisville, KY, USA). N,N-Diisopropylethylamine (DIPEA) was obtained from Fluka (Buchs, Switzerland). O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium tetrafluoroborate (TBTU) and triisopropylsilane (TIS) purchased from Sigma–Aldrich

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(St. Louis, MO, USA). 1,2-ethanedithiol (EDT), Trifluoroacetic acid (TFA) and other reagents were purchased from Merck (Darmstadt, Germany).

Peptide synthesis

The synthesis of peptides was carried out manually with Rink amide resin using the solid-phase synthesis method. O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium tetrafluoroborate (TBTU) and N,N-diisopropylethylamine (DIPEA) in dimethylformamide (DMF) were used as coupling and activating reagents, respectively. Fmoc protecting group was removed from the resin and amino acids by addition of a solution of 20% (v/v) Piperidine/DMF. The Kaiser test was performed to monitor deprotection of peptides in solid phase peptide synthesis. A cleavage cocktail (TFA/H_2O/TIS/EDT: 94 /2.5 /2.5 /1) was used for side-chain deprotection and the final cleavage of the peptide from the Rink amide resin. Crude peptides were precipitated by cold diethyl ether under vigorous stirring. The peptides containing the cleavage cocktail and Et_2O were centrifuged at 4000 rpm for three minutes, followed by decantation in order to obtain the solid peptide precipitate.[17,18]

FITC Labeling of Peptides

A solution of 1.1 equivalent of FITC in pyridine/DMF/DCM (12:7:5) was prepared and incubated with the deprotected peptide for 12 h under gentle mixing conditions.[19] The completion of the reaction was confirmed by the Kaiser test. The obtained FITC-peptide was cleaved from resin with the cleavage cocktail and was precipitated by diethyl ether.

Cell Culture

The human breast cancer cell line MCF-7 was purchased from the Pasteur Institute (Tehran, Iran). The cells were grown on 25 cm^2 cell culture flasks with RPMI-1640 medium with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), sodium pyruvate (1 mM), penicillinG100 U/ml and streptomycin 100 µg/ml (AppliChem, Darmstadt, Germany) in a humidified atmosphere of 5% CO_2, 95% air at 37 °C.

Cytotoxicity analysis

The Mcf-7 cells were seeded in 200 µL of medium per well in 96-well plates 24 h prior to the experiment. The old medium was replaced by different concentrations of peptides synthesized in serum containing medium and plates incubated for 24–72 h at 37°C in a humidified atmosphere of 5% CO2. Cell viability was then determined by measuring the color intensity of the formazan solution at 570nm using an ELISA reader (Bioteck Instruments, Wmooski, VT, USA).

The percentage of inhibition of proliferation was calculated as follows:

$$\text{Inhibition of proliferation } \% = 100 \times \frac{\text{OD570(cells treated with peptide)} - \text{OD570(medium control)}}{\text{OD570(cells control)} - \text{OD570(medium control)}}$$

Fluorescent microscopy

The human breast cancer cell line MCF-7 was cultured in 6-well plates (200×10^3 cells/well) for 24 h in an incubator at 37°C in a 5% CO_2 environment. The Cells were treated with 15 µM concentration of R2W4R2-FITC, $W_2R_4W_2$- FITC, $W_3R_4W_3$-FITC and $R_3W_4R_3$-FITC. After 1 h incubation at 37 °C, the medium containing the peptide was removed. The cells were washed with PBS and examined with an emission filter of 530 nm BP (green emission) using an Olympus IX81 fluorescence microscope (Olympus Optical Co., Ltd., Tokyo, Japan).

Flow cytometry assay

Flow cytometry was used to determine the percentage of labelled cells and compared with control cells. MCF-7 cells were treated with 5 µM, 15 µM and 25 µM concentration of peptides in separate experiments for 1 h at 37°C and the cells were washed twice with phosphate buffered saline. However, because flow cytometry does not discriminate between cell membrane-bound and internalized FITC-peptides, the cells were treated with trypsin/EDTA (0.25% trypsin and 0.02% EDTA for 5 min) to minimize any contribution of peptides attached to the outer surface of the cell membrane.[20]

Mean fluorescence ratios were determined for 10,000 cells sampled after the addition of peptides. Fluorescence ratios were calculated in arbitrary units set to a value of 100 for MCF-7 cells. The results were expressed as a percentage of gated populations and data were analysed using control cells. Flow cytometry was carried out using FACSCalibur (Becton Dickinson, San Jose, CA, USA) applying an argon laser. Excitation occurred at 488 nm for FITC.

Peptide secondary structure

Structure prediction of synthesized peptides was performed to determine their secondary structure. We applied the GOR V algorithm in order to explain the different levels of uptake by MCF-7 cells. This method has high accuracy (Q3=73.5%) with a wide range of amino acid residues for predicting the secondary structures of peptides.[21] It is based on the use of information theory and Bayesian statistics for predicting the secondary structure of peptides and proteins,[22] by analysis and comparison of an input sequence with 513 proteins, to calculate the helix, sheet and coil probabilities at each residue position.[23] The GOR V server uses the FASTA sequence format and calculates the probability helix (α-helix), extended (β-sheet) and

coil structures. The probabilities are then normalized to 0 and 1 using the following equation:[23,24]

$$P_H+P_E+P_C=1$$

The GOR V server is available online at http://gor.bb.iastate.edu (the Plant Sciences Institute at Iowa State University).

Helical wheel projection and thermodynamic parameters

Helical wheel projection illustrates the correct angular displacement from one amino acid to another amino acid in a cylindrical plot of the α-helix structure.[25] The Wimley-White is one of the most common scales that can be used for free energy calculation of peptides. This value is provided by the free-energy contributions of the side chains and backbone of peptides.[26] The free energy of peptides may be used to ascertain the feasibility of peptide transport across the cell membrane. Helical wheel projection, estimation of the transfer free energy and the total hydrophobic moment of peptides were performed by Membrane Protein Explorer (MPEx 3.2), free software provided by the Stephen White Laboratory at the University of California (Irvine, CA).

Results and Discussion

Cytotoxicity in cell culture

The cell viability of the human breast cancer cell line MCF-7 are summarized in Figure 1. Compared to control, the cells underwent reduced proliferation following treatment with $R_2W_4R_2$. All of the peptides had cytotoxic effects at concentrations of 25μM and 50 μM, following a 72- hour incubation period. The cell toxicity was dose and time dependent.

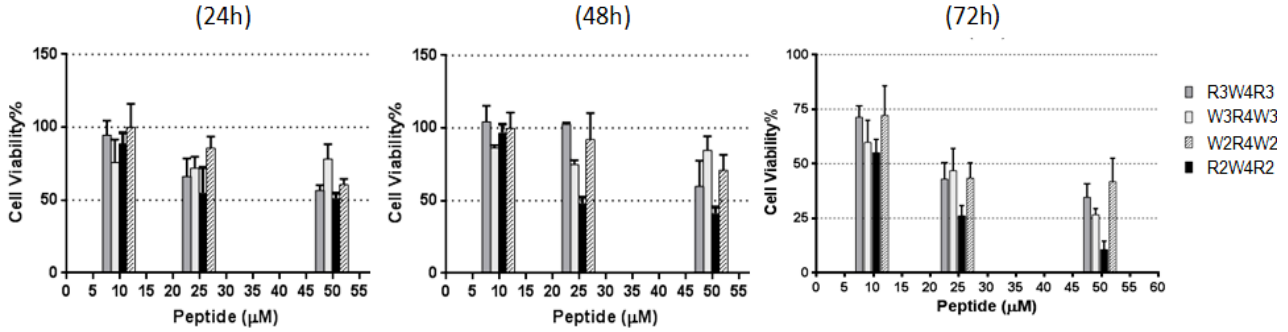


Figure 1. Cytotoxic effects of $R_3W_4R_3$, $R_2W_4R_2$, $W_3R_4W_3$, $W_2R_4W_2$. The peptides were incubated with 10^4 cells in 5, 25, and 50 μM concentrations for 24 h, 48h, and 72h. Each value is the mean ± SD of three separate determinations

Cellular Uptake and labeling

Increasing the concentration of all peptides resulted in an increased population of FITC-labelled cells (Figure 2). This effect was relatively linear in the concentration range of 5, 15 and 25μM of the peptides. The major difference was observed when comparing the two peptides containing four and six tryptophans at both ends. Accumulation of both $R_2W_4R_2$ and $R_3W_4R_3$ in MCF-7 was suitable. However the mean cellular uptake of $W_2R_4W_2$ and $W_3R_4W_3$ in 15μM concentrations of peptides was 2.52 and 7.18-fold less than that of $R_3W_4R_3$ respectively. According to the results, there was no significant difference between penetration of $R_2W_4R_2$ and $R_3W_4R_3$ sequences. It therefore appears that the location of tryptophan and arginine residues have a dramatic effect on cellular uptake. It seems that the number of tryptophan residues in $W_2R_4W_2$ and $W_3R_4W_3$ sequences affects the cellular uptake (Figure 2 and 3).

Secondary structure prediction

The results of prediction of secondary structure and the graphical model are shown in Table 1. According to the results, for $W_2R_4W_2$ sequence, coil structure is dominant with no α-helix structure. However, in other sequences, a high probability of α-helix formation was indicated. The amphipathic α-helical is important for the effective transportation of peptides across bilayer lipid membranes. It has been suggested that this structure leads to pore formation followed by peptide insertion into the cell membrane.[27] No relevance between the α-helix structure and cellular uptake of $W_2R_4W_2$ and $W_3R_4W_3$ sequences was observed.

Helical wheel projection and *total hydrophobic moment*

It has been suggested that the cellular uptake efficiency of peptide is dependent on the regular arrangement of hydrophobic and hydrophilic amino acid residues in one side of the helical wheel projection.[11,28] The helical wheel projection for $R_2W_4R_2$, $R_3W_4R_3$ and $W_3R_4W_3$ sequences are shown in (Figure 4). According to this projection, these peptides do not show any regular structure. Therefore, it cannot be used to explain differences in cellular uptake of the peptides.

The hydrophobic moment was performed to explain the high permeability of some irregular hydrophobic and hydrophilic residues in the α-helix structure, such as the amphiphilic peptide melittin.[29] The results of the total hydrophobic moment (μH) are shown in Table 2. Despite the fact that these peptides had the same value for the total hydrophobic moment, $W_3R_4W_3$ had much lower cellular uptake activity than the other peptides studied in this study.

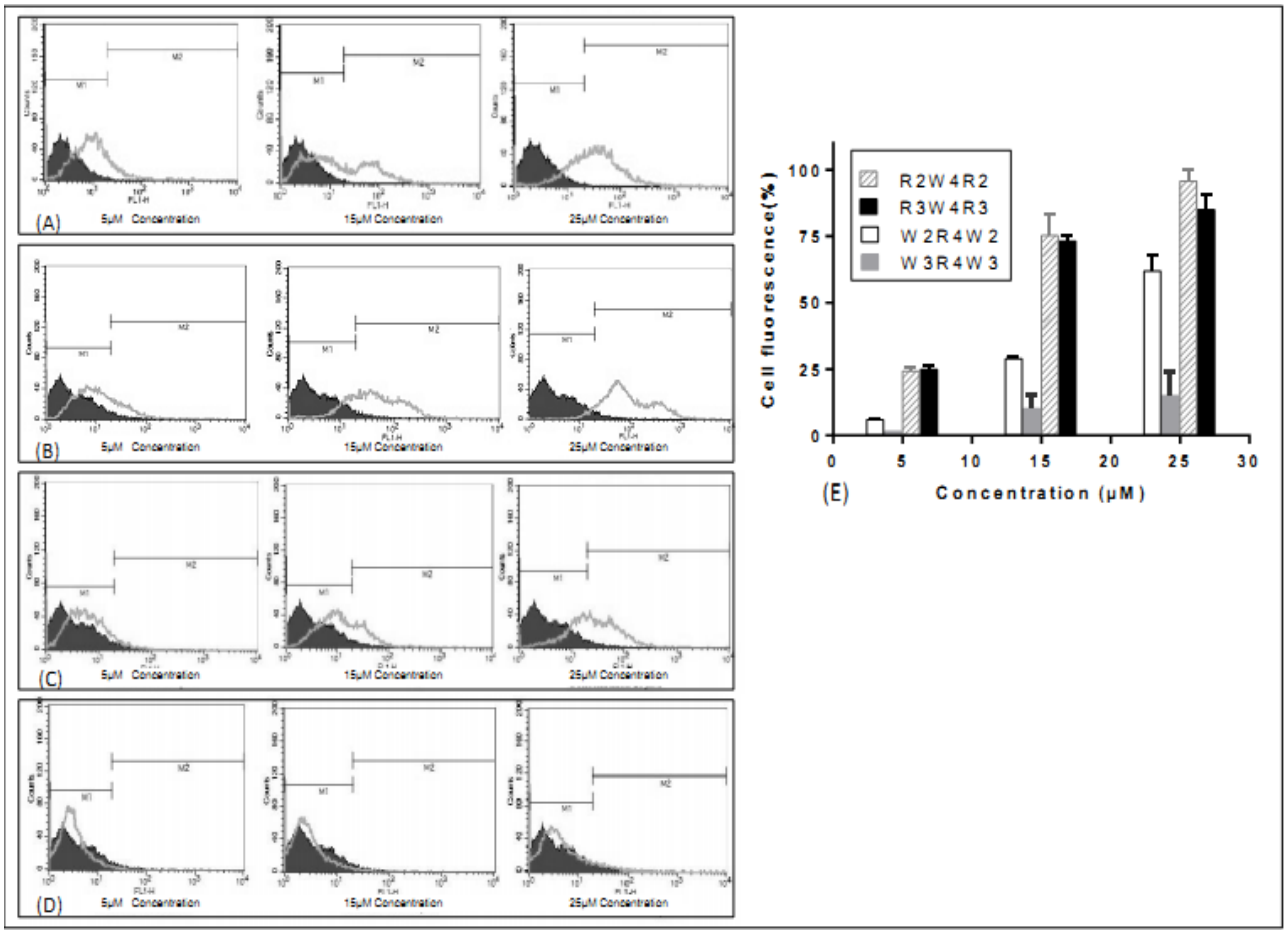


Figure 2. Cellular uptake of FITC-labeled peptides in live MCF-7 cells after incubation for 1 h at 37 °C. The uptake values of intensity are seen for $R_2W_4R_2$ (A) and $R_3W_4R_3$ (B), followed by, in order of decreasing intensity, $W_2R_4W_2$ (C), $W_3R_4W_3$ (D). (E) A comparison of the cellular uptake of peptides in 5, 15, and 25μM concentrations. The results are expressed as a percentage of gated populations. Each value is the mean ± SD of two separate determinations.

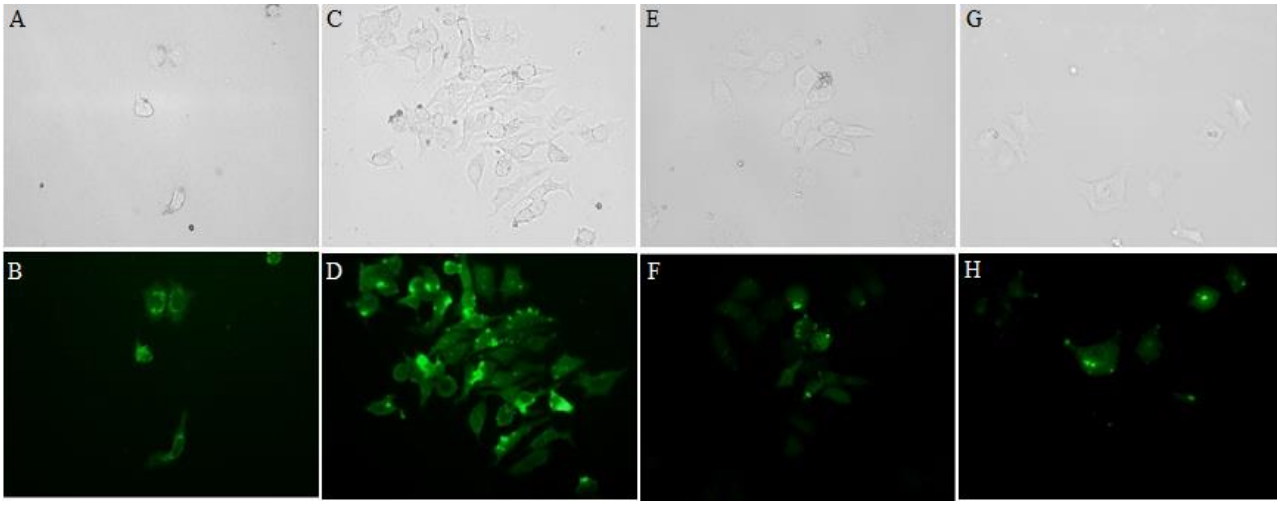
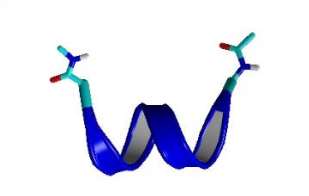
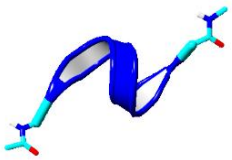


Figure 3. Fluorescence microscopy, visualization of FITC- labeled,$W_3R_4W_3$(G,H), $R_3W_4R_3$(C,D), $R_2W_4R_2$(A,B), $W_2R_4W_2$(E,F) in MCF-7 cells. Bright field (top) and fluorescence (bottom) microscopy of MCF-7 cells. Live cells were treated with 15 μM of peptides for 1h at 37°C.

Table 1. Prediction of secondary structural parameters of peptides, and graphical model

Sequence	Sequence index	Helix probability	Sheet probability	Coil probability	GOR V prediction	Graphical model
WWRRRRWW	1 W	0.114	0.129	0.758	C*	
	2 W	0.257	0.168	0.575	C	
	3 R	0.438	0.237	0.324	C	
	4 R	0.655	0.172	0.173	C	
	5 R	0.632	0.178	0.190	C	
	6 R	0.427	0.221	0.352	C	
	7 W	0.310	0.162	0.528	C	
	8 W	0.113	0.127	0.760	C	
WWWRRRRWWW	1 W	0.175	0.135	0.689	C	
	2 W	0.312	0.151	0.537	C	
	3 W	0.488	0.217	0.296	H**	
	4 R	0.655	0.200	0.145	H	
	5 R	0.655	0.172	0.173	H	
	6 R	0.632	0.178	0.190	H	
	7 R	0.549	0.253	0.197	H	
	8 W	0.421	0.245	0.334	H	
	9 W	0.247	0.166	0.586	C	
	10 W	0.123	0.133	0.743	C	
RRWWWWRR	1 R	0.102	0.170	0.728	C	
	2 R	0.246	0.241	0.513	C	
	3 W	0.399	0.267	0.334	H	
	4 W	0.543	0.286	0.171	H	
	5 W	0.595	0.255	0.150	H	
	6 W	0.512	0.265	0.222	H	
	7 R	0.387	0.213	0.400	C	
	8 R	0.234	0.150	0.616	C	
RRRWWWWRRR	1 R	0.074	0.146	0.779	C	
	2 R	0.179	0.268	0.553	C	
	3 R	0.393	0.267	0.340	H	
	4 W	0.531	0.291	0.178	H	
	5 W	0.543	0.286	0.171	H	
	6 W	0.595	0.255	0.150	H	
	7 W	0.648	0.224	0.128	H	
	8 R	0.542	0.229	0.229	H	
	9 R	0.435	0.204	0.360	H	
	10 R	0.213	0.137	0.649	C	

**α-helix (H),* coil (C).

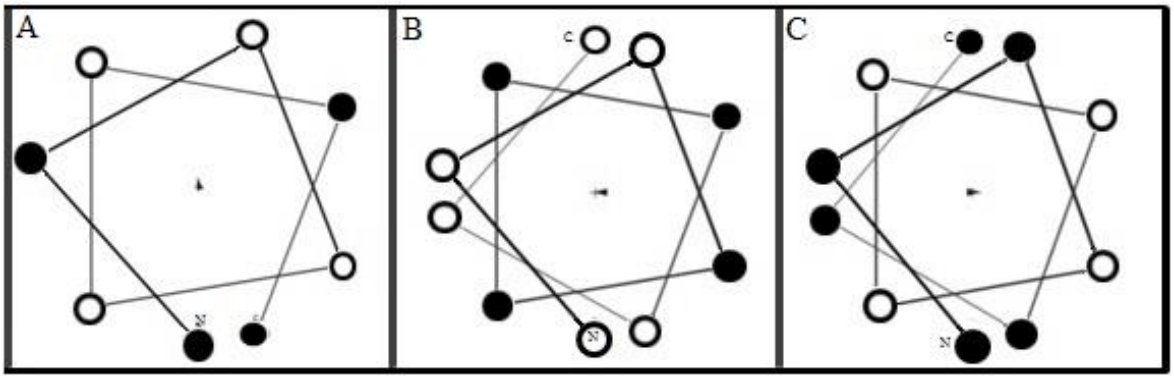


Figure 4. Illustration of sequences in helical wheel projection of $W_3R_4W_3$ (B), $R_2W_4R_2$ (A), and $R_3W_4R_3$ (C) generated using the program MPEx 3.2. Black symbols represent basics and hydrophilic residues (Arg), white, aromatics and hydrophobic residues (Trp).

Wimley-White scales

It is believed that difference between octanol hydrophobicity scale and interfacial hydrophobicity scale can be an effective parameter to predict behavior of peptides to penetrate the cell membrane. According to the research conducted in this field, peptides with $\Delta G°_{Oct\text{-}w} \leq 20$ kcal/mol^{-1} would be good candidates for penetrating the cell membrane.[30] Based on the values obtained from the W.W scale (Table 2) and flow cytometry data, $\Delta G°_{Oct\text{-}w}$ does not appear to be a suitable parameter for predicting cell penetrating peptides, since $\Delta G°_{Oct\text{-}w}$ of all peptides were less than 20 kcal/mol^{-1}.

Table 2. Hydrophobicity scales and the total hydrophobic moment

Sequence	NH2-R2W4R2-CONH2	NH2-R3W4R3-CONH2	NH2-W3R4W3-CONH2	NH2-W2R4W2-CONH2
Total. Hyd. Moment (μH)*	3.26	3.26	3.49	3.49
Wimley-White Scale(Oct-If)** (kcal mol^{-1})	9.51	11.51	9.03	9.51
Wimley-White Scale (Oct)*** (kcal mol^{-1})	3.18	6.8	-1	3.18

*Total. Hyd. Moment= the total hydrophobic moment
**Wimley–White Scale (Oct-If) ==difference between the Wimley–White whole-residue octanol hydrophobicity scale and interfacial hydrophobicity scale.
***Wimley–White Scale (Oct) = Wimley–White whole-residue octanol hydrophobicity scale

Another parameter used to predict the penetration efficiency of peptides is the transfer free energy values of peptide from water to membrane. The amount of energy required for transferring a random-coil and α-helix insertion into the hydrophobic core of the cell membrane is +1.15 kcal mol^{-1} ($\Delta G_{Oct} \geq +1.15$ kcal mol^{-1}).[31] According to the data in Table 2, $R_2W_4R_2$, $R_3W_4R_3$ and $W_2R_4W_2$ have enough energy for penetration from water to membrane. The data also shows that $W_3R_4W_3$ with lowest cellular uptake has not sufficient free energy value. It seems that uptake efficiency and transfer free energy values correlate with each other and this parameter may be used to explain differences in uptake of the peptides into the MCF-7 cells (Figure 5).

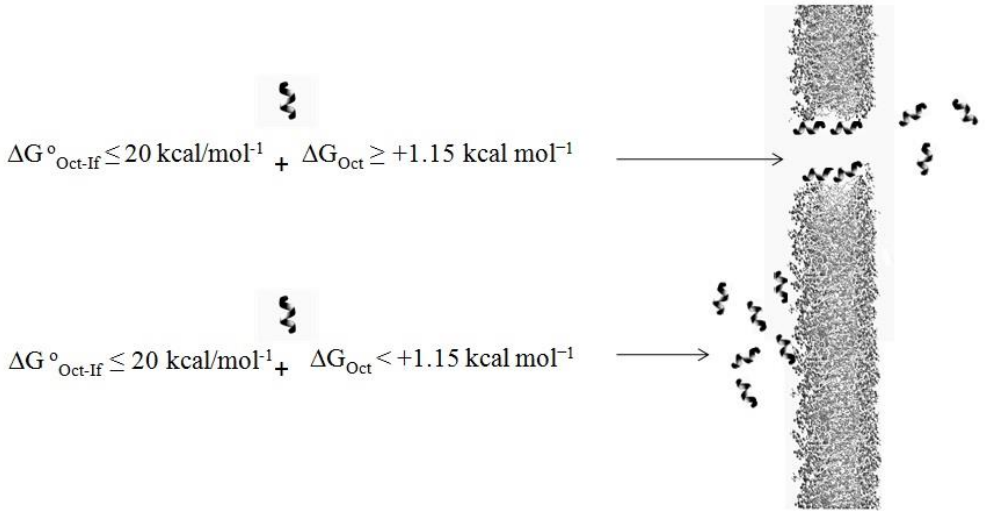


Figure 5. Effect of thermodynamic parameters for the transfer of peptide from water to the intracellular space.

Conclusion

In this study, four amphipathic peptides containing tryptophan and arginine amino acid residues were synthesized to assess the importance of different parameters in their cell uptake. The flow cytometry results showed different cellular uptake efficiency. Various thermodynamic and structural parameters that might influence peptide transport through the cell membrane were investigated. The secondary structure and the helical wheel projection failed to describe the differences in cellular uptake of the peptides. On the other hand it seems that the uptake efficiency correlates well with the transfer free energy values and this parameter may be used to describe the different uptake efficiency of the cell penetrating peptides into MCF-7 cells.

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Ethical Issues

Not applicable.

Conflict of Interest

The authors report no conflicts of interest.

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