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Correlation between the secondary structure and surface activity of β-sheet forming cationic amphiphilic peptides and their anticancer activity

Roja Hadianamrei[1], Mhd Anas Tomeh[1], Stephen Brown[3], Jiqian Wang[4], Xiubo Zhao[1,2*]

[1] Department of Chemical and Biological Engineering, University of Sheffield, S1 3JD, UK.

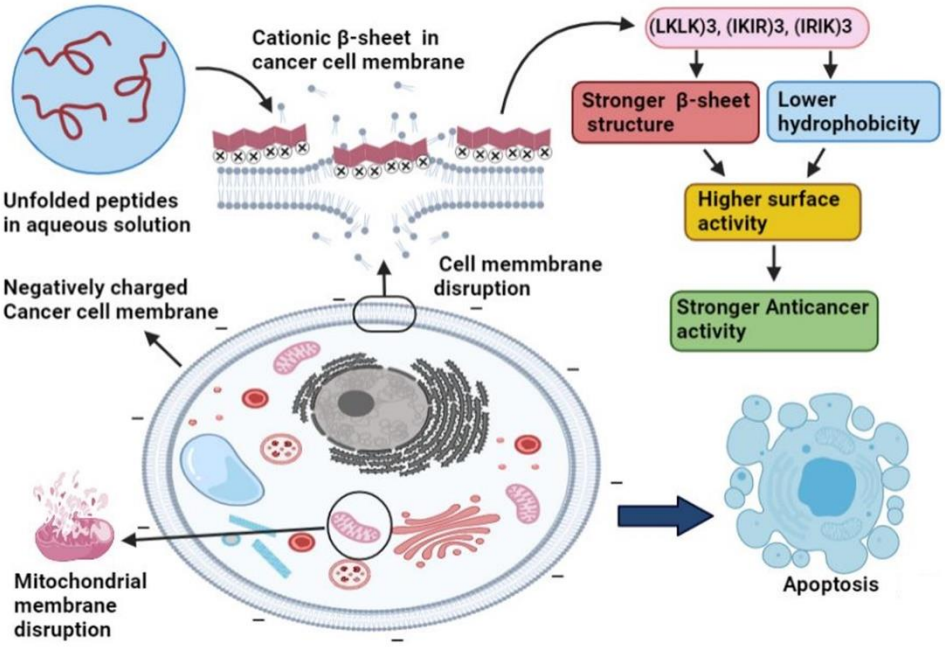
[2] School of Pharmacy, Changzhou University, Changzhou 213164, China.

[3] Department of Biomedical Science, University of Sheffield, S10 2TN, UK.

[4] Centre for Bioengineering and Biotechnology, China University of Petroleum (East China), Qingdao 266555, China.

*Corresponding author: E-mail: xiubo.zhao@sheffield.ac.uk Tel: +44-(0)-114-222-8256

Graphral Abstract



Abstract

Cancer is one of the main causes of death worldwide. The current cancer treatment strategies often lack selectivity for cancer cells resulting in dose-limiting adverse effects and reduced quality of life. Recently, anticancer peptides (ACPs) have emerged as an alternative treatment with higher selectivity, less adverse effects, and lower propensity for drug resistance. However, most of the current studies on the ACPs is focused on α-helical ACPs and there is lack of systematic studies on β-sheet forming ACPs. Herein we report the development of a new series of rationally designed short cationic amphiphilic β-sheet forming ACPs and their structure activity relationship. The peptides had the general formula $(XY_1XY_2)_3$, with X representing hydrophobic amino acids (isoleucine (I) or leucine (L)), Y_1 and Y_2 representing cationic amino acids (arginine (R) or lysine (K)). The cytotoxicity of the designed ACPs in HCT 116 colorectal cancer, HeLa cervical cancer and human dermal fibroblast cells was assessed by MTT test. The physicochemical properties of the peptides were characterized by various techniques including RP-HPLC, LC-MS, and Circular Dichroism (CD) spectroscopy. The surface activity of the peptides at the air-water interface and their interaction with the lipid monolayers as models for cell membranes were studied by Langmuir trough. The peptides consisting of I with R and K had selective anticancer activity while the combination of L and R diminished the anticancer activity of the peptides but rendered them more toxic to HDFs. The anticancer activity of the peptides was directed by their surface activity (amphiphilicity) and their secondary structure in hydrophobic surfaces including cancer cell membranes. The selectivity of the peptides for cancer cells was a result of their higher penetration into cancer cell membranes compared to normal cell membranes. The peptides exerted their anticancer activity by disrupting the mitochondrial membranes and eventually apoptosis. The results presented in this study provide an insight into the structure-activity relationship of this class of ACPs which can be employed as guidance to design new ACPs with improved anticancer activity and lower toxicity against normal cells.

Keywords: Anticancer peptides; cationic amphiphilic peptides; beta sheet peptides; cervical cancer; colorectal cancer; surface activity.

1. Introduction

Cancer is caused by genetic mutations in the "driver genes" which renders the cancer cells capable of evading growth suppression, resisting cell death, and metastasizing [1-4]. It has become a major global health concern and one of the main causes of death worldwide. The currently available antineoplastic agents often lack selectivity for cancer cells and cause damage to healthy tissues leading to adverse effects that could be dose-limiting or reduce the patient's quality of life [5-13]. The development of multidrug resistance (MDR) is another major concern associated with conventional anticancer drugs [5, 14]. Although immunotherapy with monoclonal antibodies, immune checkpoint inhibitors and modified immune cells [3, 15-24] more selectively targets the cancer cells, it is still associated with dose-limiting adverse effects and in some cases lethal hypersensitivity reactions [24-29]. Furthermore, the complexity and high costs of manufacturing place immunotherapy drugs among the most expensive drugs in the market [24, 27, 30, 31]. Hence, there is still a quest for the development of new anticancer drugs with high selectivity for cancer cells, low propensity for drug resistance and low production costs.

Recently, anticancer peptides (ACPs) have been introduced as an alternative to conventional antineoplastic agents. Naturally occurring host defence peptides with antimicrobial or antifungal activity are found in different organisms including plants, insects, amphibians, and mammals [32-36]. Some of these peptides also possess selective anticancer activity [35-37]. Due to their smaller size and higher solubility compared to monoclonal antibodies and checkpoint inhibitors, the ACPs enjoy better pharmacokinetics and higher cellular uptake which could enhance their potency and efficacy [38].

It is well evidenced that the higher selectivity of the ACPs for cancer cells compared to normal cells lies in the higher affinity of these cationic peptides for the anionic membrane of the cancer cells compared to the zwitterionic membrane of the normal cells [32-37, 39]. The more negative charge of the cancer cell membranes is due to the presence of negatively charged phospholipids phosphatidylserine (PS) and higher abundance of anionic molecules such as heparan sulfates and O-glycosylated mucins[32-37, 39]. Since the ACPs target the cancer cell membranes, their selectivity for cancer cells is less affected by the tumour heterogeneity and also, they are less prone to drug resistance which is one of their advantages over the other types of anticancer agents [33, 35, 36]. Over the last decade, more research has been directed towards developing synthetic ACPs to reduce their production costs, improve their physicochemical properties, enhance their resistance to enzymatic proteolysis, and reduce their risk of immunogenicity [35, 40-48]. Despite the large amount of literature on the structure activity relationship (SAR) of the α-helical antimicrobial and anticancer peptides, there are very few studies on β-sheet forming peptides and most of these studies have focused on the antimicrobial activity [49-53] and there are very few studies on SAR of β-sheet forming anticancer peptides [52, 54]. Hence, there is the need for systematic studies correlating the structure and physicochemical properties of the β-sheet forming peptides to their potential anticancer activity and selectivity.

Herein we report the development of a new series of de novo designed β-sheet forming anticancer peptides with selective anticancer activity against colorectal and cervical cancer cells. The cytotoxicity of the designed peptides in cancer cells and normal cells was assessed with regard to their physicochemical properties. Furthermore, the tendency of the peptides to penetrate into different types of lipid mono and bilayers mimicking normal and cancer cell membranes were studied to provide an insight into the mechanism of cell selectivity of these peptides. The general formula for this series of peptides is $(XY_1XY_2)_3$, with X representing hydrophobic amino acids (isoleucine (I) or leucine (L)), Y_1 and Y_2 representing cationic amino acids (arginine (R) or lysine (K)). The peptides were designed using de novo minimalist design approach based on the common occurrence of amphipathic dyad repeats in β-sheet forming peptides which allows for orientation of alternating residues toward alternating faces of the β-sheet [49, 51, 55]. The choice of the hydrophobic amino acids was based on their high propensity for forming β-sheets and their high occurrence in β-sheets in naturally occurring proteins [56-59]. The cationic (hydrophilic) amino acids were chosen based on their high abundance in the naturally occurring β-sheet forming peptides [59]. The peptides were amidated at the C-terminal to enhance their anticancer activity by increasing the positive charge density [60-62]. The current study also uses a systematic approach by keeping the length and the net positive charge of the peptides constant while changing the amino acid combination in the repeat unit to investigate the effect of such structural changes on the β-sheet forming tendency, the anticancer activity, and cell selectivity of the resulting peptides. Although this combination of hydrophobic and cationic amino acids has been previously used for developing β-sheet forming antimicrobial peptides with different sizes and sequences [49, 51], to the best of our knowledge there have been no reports on anticancer peptides with these structures. Furthermore, while the structure-activity relationship of the α-helical AMPs and ACPs have been widely studied, there is very limited literature data on the structure activity relationship of the β-sheet forming AMPs and none on the β-sheet forming ACPs.

2. Materials and methods

Materials

The peptides were synthesized by Fmoc solid-phase synthesis using a commercial CEM Liberty peptide synthesizer. The synthesis was carried out from the C-terminus to the N-terminus on the Rink amide MBHA resin, thus producing C-terminally amidated peptides. The peptides were purified by cold ether precipitation eight times to reach the purity of >95%, followed by lyophilization for 2 days. The peptide solutions were prepared by dissolving the lyophilized peptide powders in Milli-Q water (Millipore Reagent Water System, USA) and their pH was adjusted to the desirable range using sodium hydroxide. All the chemicals, reagents and organic solvents were sourced from Merck (Sigma Aldrich), UK, with analytical grade. Rink amide-methylbenzhydrylamine hydrochloride salt (MBHA) resin, and 9-fluorenyl-methoxycarbonyl (Fmoc) protected amino acids were bought from GL Biochem Ltd (Shanghai, China).1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DPPG) were purchased from Avanti Polar Lipids

(USA). The Dulbecco's Modified Eagle Medium (DMEM), Phosphate buffered saline (PBS), Fetal bovine serum (FBS), Trypsin, Penicillin and streptomycin were sourced from GIBCO (Thermo Fisher Scientific, UK). JC-1 mitochondrial probe (Invitrogen™) was sourced from Invitrogen (Thermo Fisher Scientific, UK).

Determination of peptide sequence and purity

The peptide sequences and molecular weights were verified by Liquid chromatography-Mass spectrometry (LC-MS) (QExactive HF, Thermo Fisher™). The full MS scan from m/z= 375-1500 was acquired in the Orbitrap at a resolution of 120,000. Subsequent fragmentation was Top 2 in the HCD cell, with detection of ions in the Orbitrap using centroid mode, with a resolution of 30,000. The purity of the peptides was checked by reverse phase high performance liquid chromatography (RP-HPLC) using Waters 2695 HPLC system, with Waters 2487 UV/Visible detector, and Xbridge C18 column (4.6 x 250 mm). The mobile phase consisted of Acetonitrile and Trifluoroacetic acid (TFA) in water (0.1% V/V), with a gradient of 5% to 95% Acetonitrile over 20 mins at a flow rate of 1 mL/min.

Determination of peptide hydrophobicity

The hydrophobicity of the designed peptides was determined both theoretically using the Eisenberg method [63, 64] and experimentally using RP-HPLC retention times. The mean hydrophobicity value for each peptide was calculated using the following equation:

$$< H > = \left(\sum_{i=1}^{N} H_i\right)/N \qquad \textit{Eq. 1.}$$

Where <*H*> is the mean hydrophobicity of the peptide sequence, H_i is the hydrophobicity of each amino acid in the peptide sequence and *N* is the number of amino acid residues [63, 64]. The hydrophobicity of amino acids was based on their octanol-water partition coefficients as reported by Fauchère and Pliska [65, 66].

Determination of peptide secondary structure

The secondary structure of the peptides was determined by Circular Dichroism (CD) spectroscopy using a Jasco J-810 spectropolarimeter and a quartz cell with 1 cm path length. The samples were scanned in the far UV (λ= 190-240 nm), at a scanning speed of 100 nm/min and a fixed peptide concentration (10 µM). The CD measurements were performed on peptides in aqueous solution and in three different types of curved lipid bilayers: Sodium dodecyl sulfate (SDS) micelles, DPPG small unilamellar vesicles (SUVs) and DPPC SUVs. All measurements were performed in triplicate and the data were reported as the average of the three repeats. The mean residue molar ellipticity was calculated using the following equation:

$$\theta_M = \frac{\theta_{Obs}}{10} \cdot \frac{M_{RW}}{c \cdot l} \qquad \textit{Eq. 2.}$$

Where Θ_M is residue molar ellipticity (deg.cm^2.dmol^{-1}), Θ_{obs} is the observed ellipticity at a given wavelength (mdeg), M_{RW} is residue molecular weight obtained by dividing the molecular weight of

the peptide by the number of amino acid residues, *c* is the peptide concentration (mg/mL), and *l* is the path length of the cell (cm) [49, 50, 67-69].

Preparation of lipid vesicles

The DPPC and DPPG SUVs were prepared by thin-film hydration method. Briefly, a thin lipid film was produced from lipid solution in chloroform (at a concentration of 2 mg/mL) by solvent evaporation using a rotary evaporator (Heidolph Instruments GmbH & CO). Rehydration of the lipid film with phosphate buffer (pH=7.4) produced SUVs. The SUVs were then homogenized and brought to the desired size (≤ 200 nm) by extrusion through Avanti mini-Extruder (Avanti Polar Lipids, USA) containing a polycarbonate membrane with a pore size of 200 nm. The SDS micelles were simply prepared by dissolving SDS powder in Milli-Q water at a concentration of 25 mM. The size of the lipid vesicles was measured by Dynamic light scattering (ZetaPALS, Brookhaven instruments corporation) and reported as the average of 6 scans.

Surface activity and interaction of the peptides with lipid monolayers

The surface pressure measurements were performed using a Langmuir trough (NIMA technology Ltd, Coventry, UK), with a 3 ml built-in Teflon trough filled with PBS (pH=7.4) and a Wilhelmy plate attached to the pressure sensor. The peptide solution at different concentrations (10-40 µM) was injected underneath the buffer surface using a Hamilton microsyringe and the changes to the surface pressure at the air-water interface were recorded as a function of time for 2 h. The surface pressure was obtained by calculating the difference between the initial surface tension of the pure water and the final surface tension following adsorption of the peptides at the air/water interface:

$$\pi = \gamma_0 - \gamma \qquad \textit{Eq. 3.}$$

Where π is the surface pressure, γ_0 is the initial surface tension of pure water and γ is the final surface tension [41, 70].

The interaction of the peptides with the lipid monolayers was studied by monitoring the changes to the surface pressure of DPPG and DPPC lipid monolayers upon contact with the peptide solution. The lipid monolayers were formed by spreading the lipid solution in chloroform (0.5 mg/mL) at the air-buffer interface using a Hamilton microsyringe and allowing for the solvent to evaporate (20 min). The peptide solution was injected into the subphase (at a final concentration of 20 µM) and the changes to the surface pressure over time were monitored for 2 h. The initial pressure of the lipid monolayer was set to 28 mN/m which is close to the average cell membrane resting pressure [61, 62, 71]. All the measurements were performed in triplicate and the values were reported as the average of the three repeats.

Cytotoxicity tests

The cytotoxicity tests were performed in three different cell lines: HCT 116 colorectal adenocarcinoma cells, HeLa cervical cancer cells, and Human dermal fibroblasts (HDFs). The cells

were cultured in DMEM enriched with 10% FBS and 1% antibiotic (100 U/mL penicillin and 100 µg/mL streptomycin) at 37 °C under 5% CO_2. For the cytotoxicity tests, the cells were cultured in 96 well plates at a seeding density of 4000 cells/well and incubated with different concentrations of the peptide solutions for 72 h. The cell viability was assessed by MTT assay following standard protocols. Briefly, 10 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL) was added to each well and incubated at 37 °C for 4 h. Subsequently, the media was removed and replaced with Dimethyl Sulfoxide (DMSO). The plates were shaken for 15 min to allow for complete dissolution of the formazan dye and then the absorbance of formazan at 590 nm was measured using a microplate reader (Varioskan Flash™, Thermo Fisher Scientific). The relative cell viability was determined with respect to the untreated controls. All experiments were repeated 6 times and the values were reported as Mean ± SE of the 6 replicates.

Mitochondrial damage tests

The ability of the designed anticancer peptides to damage the mitochondrial membrane was evaluated using JC-1 mitochondrial probe. The cells were cultured in 96 well plates at a seeding density of 4000 cells/well and incubated with the peptide solutions (at the concentration of 20 µM). After 72 h, the cells were stained with JC-1 following the manufacturer's protocol. Briefly, the cells were washed with PBS, immersed in fresh media containing JC-1 (10 µg/mL) and incubated at 37 ˚C for 15 min. Subsequently, the media was removed, the cells were washed with PBS, submerged in PBS and imaged with high content fluorescent automated widefield microscope (ImageXpress® Micro System, Molecular Devices, USA).

Data analysis

The quantitative data were analysed using Microsoft® Excel 2016 and GraphPad Prism 9. All data were reported Mean ± SE of the repeats. For the correlation graphs the data were subjected to linear regression analysis at 95% confidence interval ($\alpha = 0.05$) and values of $p < 0.05$ were used to determine the goodness of fit. The microscopic images were analysed using MetaXpress® software 5.3.01 (Molecular Devices, USA).

3. Results and discussion

Structure and physicochemical properties of the peptides

The molecular weights measured by LC-MS were similar to the theoretical molecular weights calculated using the online software which confirms the peptide sequences (**Table 1**). Comparing the values of mean residue hydrophobicity of different peptides in this series indicated that there was no significant difference between the hydrophobicities of the peptides that contained the same type of amino acid in their hydrophobic domain, but the isoleucine-rich peptides had slightly higher hydrophobicity than the leucine-rich counterparts. Changing the hydrophilic amino acids (arginine or lysine) in the peptide sequence seemed to have a minimal effect on their mean residue

hydrophobicity. On the contrary, the apparent hydrophobicities determined by RP-HPLC retention times revealed a different trend for hydrophobicity in this series of peptides. The leucine-rich peptides were more hydrophobic than the isoleucine-rich peptides and there was a significant increase in hydrophobicity by replacing the lysine residues with arginine residues. These observations further confirm the previously reported claims that the hydrophobicity of a peptide is not only a function of its amino acid composition or polarity but is also influenced by other factors such as peptide secondary structure. Therefore, the RP-HPLC retention time provides a more accurate measure of the peptide hydrophobicity than the mean residue hydrophobicity as it reflects the real-time interaction of the peptide with the hydrophobic surface of the HPLC stationary phase [41, 68, 72, 73].

Table 1. Sequences and physicochemical properties of the designed β-sheet forming anticancer peptides.

Peptide	Sequence	Charge	Theoretical MW[a]	Measured MW[b]	RT[c]	<H>[d]
IKIK	IKIKIKIKIKIK-NH_2	+6	1464.10	1464.12	8.6	0.405
LKLK	LKLKLKLKLKLK-NH_2	+6	1464.10	1464.12	8.9	0.355
IKIR	IKIRIKIRIKIR-NH_2	+6	1548.12	1548.14	8.8	0.400
LKLR	LKLRLKLRLKLR-NH_2	+6	1548.12	1548.14	9.2	0.350
IRIK	IRIKIRIKIRIK-NH_2	+6	1548.12	1548.14	8.8	0.400
LRLK	LRLKLRLKLRLK-NH_2	+6	1548.12	1548.14	9.2	0.350
LRLR	LRLRLRLRLRLR-NH_2	+6	1632.14	1632.16	9.5	0.345

a Theoretical molecular weights calculated using the online tool from the website "https://pep-calc.com"; b Experimental molecular weights measured by LC-MS; c HPLC retention times; d Mean residue hydrophobicity calculated by Eisenberg method using the hydrophobicity scale defined by Fauchère and Pliska.

Secondary structure of the peptides

The secondary structure of the designed anticancer peptides in different environments as determined by circular dichroism (CD) spectroscopy are presented in **Figure 1**. All peptides except IKIR and IRIK had an unfolded random coil structure in the aqueous solution as indicated by a negative peak at near 198-200 nm [49, 50, 74]. This is supposed to be because of the electrostatic repulsion between the positively charged arginine/lysine residues within the peptide molecules [49]. IKIR and IRIK on the other hand, exhibited β-sheet structures indicated by a positive peak at 197-200 nm and a negative peak at 217-218 nm [49, 50, 53]. The different behaviour of IKIR and IRIK compared to the rest of the peptides in this series is suggested to result from the interplay between the electrostatic parameter and the other pertinent factors which contribute to the peptide folding in aqueous media including steric parameter, hydrophobic parameter, amino acid side chain density, amino acid nonpolar accessible surface area, and overall amino acid packing density [75-80]. The steric parameter which reflects the bulk and branching of the amino acid side chains influences the rotational flexibility of the peptide chain and consequently its likelihood to fold into β-sheet conformation in aqueous solution [77, 78]. I has higher steric parameter than L and therefore the peptides containing I are more likely to form β-sheet structures than the peptides containing L [77, 78]. Similarly, the overall side chain density of the amino acids in the peptide sequence affects the

rotational flexibility of the peptide and the combination of amino acids in IRIK/IKIR has lower overall side chain density compared to their leucine bearing counter parts and also compared to IKIK which bestows higher rotational flexibility upon these two peptides [75, 80]. The higher hydrophobicity reported for R compared to K in several hydrophobicity scales such as the Wimley White hydrophobicity scale [75] and the Dwyer inverted hydrophobicity scale [77] further justifies the higher propensity of IRIK and IKIR for forming β-sheet conformation compared to IKIK as it has been evidenced that the higher hydrophobicity of amino acids increases their preference for forming β-sheet structures [77]. Furthermore, the lower overall accessible surface area [75, 76] and the higher overall residue packing density [79] for IKIR and IRIK compared to the other peptides in this series also justifies the formation of β-sheet structures by these two peptides in aqueous solution while other peptides in this group remain unfolded. The accessible surface area and the packing density of amino acids in a peptide or protein sequence are considered as predictors of folding of peptides and proteins [75, 76, 79].

Unlike the aqueous solution in the anionic environment of SDS micelles and DPPG SUVs all peptides adopted β-sheet structures denoted by a positive peak at 197-200 nm and a negative peak at 217-218 nm [49, 50, 53]. This conformational change is supposed to happen as a result of the electrostatic interaction between the positively charged arginine/lysine residues of the peptides and the negatively charged SDS/DPPG headgroups followed by interaction of the hydrophobic residues of the peptide with the hydrophobic tail of the SDS/DPPG [49, 53].In the zwitterionic environment of DPPC SUVs, on the other hand, the peptides showed a combination of random coil structure and β-sheet structure, indicated by a positive peak at 194-200 nm and a negative peak at 200-205 nm. This could be indicative of some regions of the peptide adopting β-sheet conformation while the other regions remaining unfolded. Alternatively, this could result from partial penetration of some peptide molecules into the DPPC lipid bilayer leading to β-sheet formation while the other peptide molecules still exist in random coil structure in the aqueous phase of the SUVs. Similar observations were reported for the antimicrobial peptide arenicin which had a mixture of β-sheet and random coil structure in the micelles of non-ionic surfactant octyl-β-D-glucopyranoside (OG) [53].

These findings are in accordance with the results from other studies with β-sheet forming antimicrobial peptides with closely related structures. For example, Ong et al [49] developed short cationic antimicrobial peptides consisting of arginine or lysine in their hydrophilic domain and valine, isoleucine, phenylalanine or tryptophan in their hydrophobic domain. The peptides had a random coil structure in water but transformed into β-sheet structure in SDS micelles [49]. Similar observations have been reported for some of the cell penetrating peptides including penetratin, MPG, and M918 which had random coil structure in water and zwitterionic DOPC vesicles but folded into β-sheet structure in anionic DOPG phospholipid vesicles [74]. Unlike the rest of the peptides, IKIR and IRIK exhibited β-sheet structure both in the anionic environments and in the neutral or zwitterionic environments. Nonetheless, they possessed a less defined β-sheet conformation in DPPC SUVs compared to DPPG SUVs and SDS micelles. This suggests that the presence of isoleucine residues in the hydrophobic domain of the peptide is more favourable for β-sheet formation than leucine

residues. Moreover, the combination of lysine and arginine residues in the hydrophilic domain of the peptide enhances the tendency for β-sheet conformation than either arginine or lysine alone.

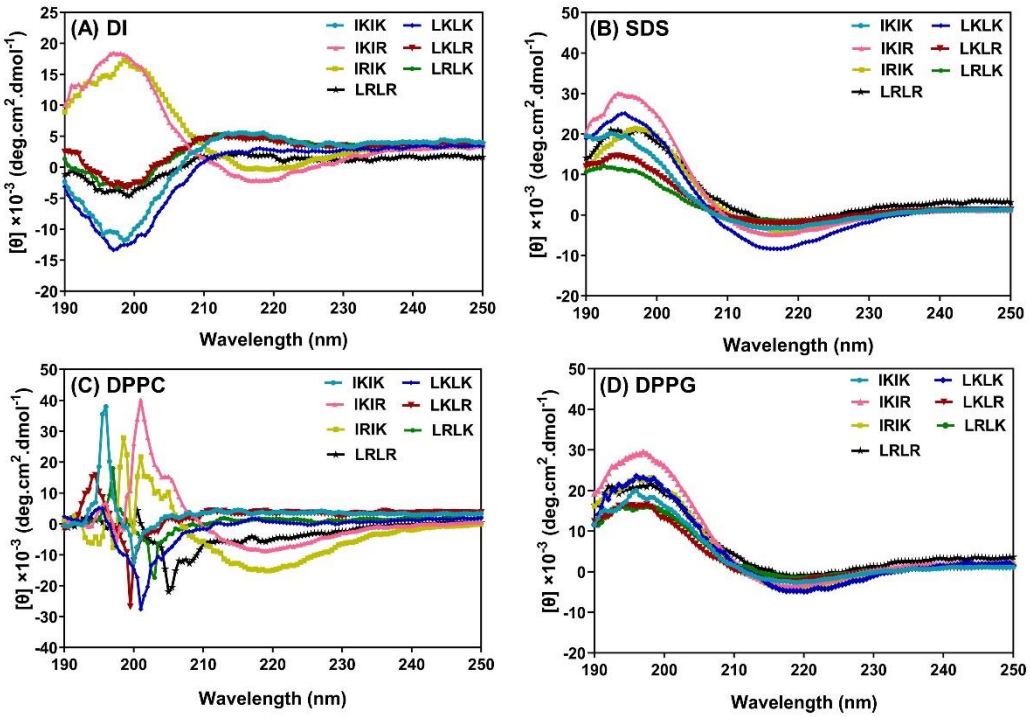


Figure 1. Circular dichroism (CD) spectra of the cationic amphiphilic peptides in DI water (**A**), SDS micelles (**B**), DPPC SUVs (**C**) and DPPG SUVs (**D**).

Cytotoxicity of the peptides in normal and cancer cells

The cytotoxicity of the designed anticancer peptides as determined by MTT assay is depicted in **Figure 2** and the values of half maximal inhibitory concentration (IC_{50}) of the peptides in different cell lines are presented in **Table 2**. As it could be inferred from these data, LKLK was the most toxic peptide in HCT 116, with the highest efficacy and potency in the experimental concentration range (IC_{50} = 14.5 ± 1.3 μM and 68.1 ± 2.1 % growth inhibition). Replacing the leucine residues of this peptide with isoleucine residues (IKIK) or replacing the lysine residues with arginine residues (LKLR and LRLK and LRLR) resulted in considerable decrease in anticancer activity against HCT 116 compared to LKLK as evidenced by the higher values of IC_{50} for these peptides. IKIR also showed considerable toxicity in HCT 116 cells whereas its leucine containing analogue, LKLR did not show significant toxicity against HCT 116 in the experimental range of concentrations.

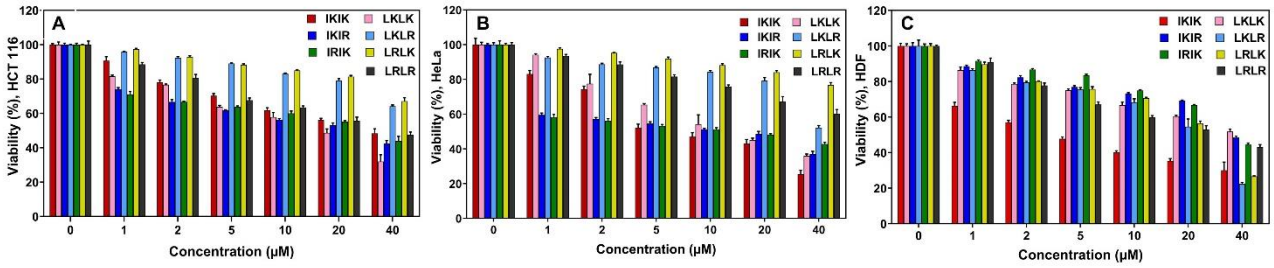


Figure 2. Cytotoxicity of the designed anticancer peptides in HCT 116 (**A**), HeLa (**B**), and HDF (**C**) as determined by MTT assay. All values were normalized compared to the untreated controls and reported as Mean ± SE of 6 repeats.

Table 2. Half maximal inhibitory concentrations (IC_{50}) of the designed anticancer peptides in different cell lines as determined by MTT assay. All values are reported as Mean ± SD of 6 replicates.

Peptide	IC_{50} (μM)		
	HCT 116	**HeLa**	**HDF**
IKIK	32.7 ± 4.5	8.9 ± 2.1	4.4 ± 0.3
LKLK	14.5 ± 1.3	17.7 ± 6.5	> 40
IKIR	20.9 ± 2.0	14.5 ± 2.3	38.6 ± 0.2
LKLR	> 40	> 40	22.5 ± 1.4
IRIK	30.1 ± 5.2	15.5 ± 1.2	34.5 ± 0.9
LRLK	> 40	> 40	24.1 ± 0.7
LRLR	31.8 ± 2.9	> 40	22.7 ± 2.4

In HeLa cells, on the other hand, IKIK was the most toxic peptide (IC_{50} = 8.9 ± 2.1 μM and 74.4 ± 1.4 % growth inhibition). LKLK, IRIK, and IKIR were also highly toxic to HeLa cells without any significant difference between them. The rest of the peptides did not exhibit any cytotoxicity against HeLa cells. In contrast to the trend observed for cytotoxicity in cancer cells, in HDF cells the peptides containing leucine in their hydrophobic domain and arginine in their hydrophilic domain (LRLR, LRLK, and LKLR) exhibited considerable cytotoxicity, whereas LKLK and IKIR had minimal toxicity against HDFs. IKIK also exhibited high toxicity against fibroblasts which was comparable to its toxicity against HeLa cells.

As it could be inferred from these data, the combination of leucine and arginine diminishes the anticancer activity of the peptides but renders them more toxic to HDFs. Overall, LKLK, IKIR, and IRIK possessed selective anticancer activity with low toxicity against HDFs. LKLK was equally toxic to HCT 116 and HeLa cells, while IKIR and IRIK favoured HeLa cells over HCT 116 cells. These data indicate the importance of testing the anticancer peptides in different cancer cell lines to investigate cancer-specific cytotoxicity. This could help in rational design of peptides with enhanced cytotoxicity against specific cancers and with reduced cytotoxicity against normal tissues. It is also noteworthy that the cell viability tests were performed in media enriched with FBS. Hence, the high anticancer activity of the peptides in the presence of FBS indicates their resistance to serum proteases.

Cytotoxicity of the peptides is influenced by their secondary structure and their hydrophobicity

The relationship between cytotoxicity of the peptides in different cell lines, their secondary structure in phospholipid vesicles and their hydrophobicity is depicted in **Figure 3.** The values of mean residue molar ellipticity at 197-200 nm were used to compare the β-sheet content of the different peptides [50, 53] and the RP-HPLC retention times were used as a measure of hydrophobicity [41, 68, 72, 73]. There was a strong correlation between the β-sheet content of the peptides in negatively charged DPPG SUVs and their anticancer activity in HCT 116 cells which suggests the same conformational changes from random coil to β-sheet may take place in the cell membrane of HCT 116 cells. On the other hand, no direct relationship was found between the β-sheet content of the peptides in DPPG SUVs and their anticancer activity against HeLa cells. Contrastingly, the anticancer activity of the peptides in HeLa cells was inversely proportional to their hydrophobicity whereas no direct relationship between the anticancer activity of the peptides in HCT 116 cells and their hydrophobicity was found. It could be inferred from these data that the cytotoxicity of the peptides against HCT 116 cells is more influenced by their secondary structure upon contact with cancer cell membranes whereas their cytotoxicity against HeLa cells is more directed by their hydrophobicity. Hence, the anticancer activity of the peptides is directed by an interplay between their secondary structure and their hydrophobicity.

Lack of a good correlation between the cytotoxicity of the peptides in HDF cells and their β-sheet content in zwitterionic DPPC SUVs suggests that the conformational changes upon interaction of the peptides with the normal cell membranes don't play a significant role in their cytotoxicity against normal cells. The only exception is IKIK which had a very high β-sheet content in DPPC SUVs and exhibited the highest toxicity against HDF cells among all of the peptides. This may be in part due to the presence of a mixture of β-sheet and random coil structures in these peptides in the zwitterionic lipid bilayers which reduces their penetration into the cell membrane compared to the complete β-sheet structure formed in anionic lipid bilayers as discussed earlier. On the other hand, with the exception of IKIK, the cytotoxicity of the peptides against HDF cells was directly proportional to their hydrophobicity and the most hydrophobic peptides were the most toxic. Hence, the cytotoxicity of the peptides against normal cells is mainly influenced by their hydrophobicity and the β-sheet secondary structure does not play an important role in it.

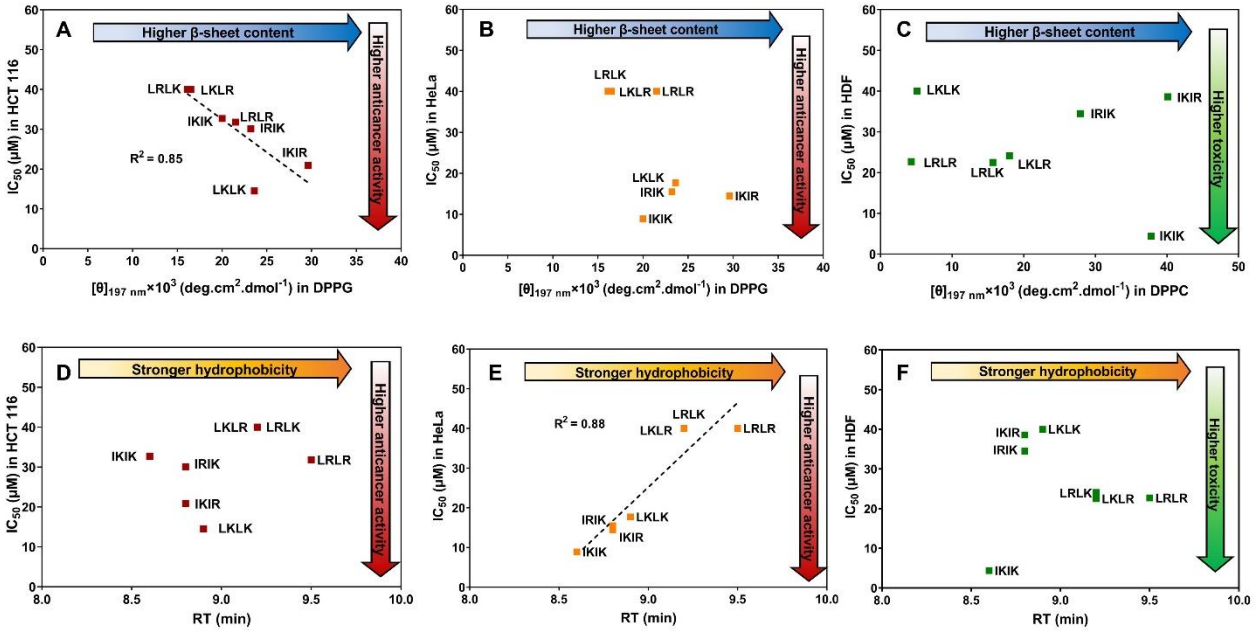


Figure 3. Relationship between the anticancer activity of the peptides in HCT 116 **(A)** and HeLa (**B**) and their β-sheet content in DPPG small unilamellar vesicles (SUVs). (**C**) Relationship between the cytotoxicity of the peptides in HDF and their β-sheet content in DPPC SUVs. Relationship between the hydrophobicity of the peptides and their cytotoxicity in HCT 116 **(D)**, HeLa **(E)**, and HDF **(F)**.

Surface activity of the peptides determines their anticancer activity

The amphiphilicity of the designed anticancer peptides was measured by their surface pressure at the air-water interface which serves as a good model for a hydrophobic/hydrophilic interface [74]. Three peptides (IKIR, IRIK and LKLK) with higher selectivity for cancer cells were studied and one peptide (LRLK) with poor anticancer activity and high toxicity in fibroblast was used as a negative control. Changes to the surface tension of pure water upon adsorption of the peptide molecules at the air-water interface, referred to as "surface pressure", is indicative of the surface activity of a given peptide [40, 41]. As it could be observed in **Figure 4A**, there was an increase in the surface pressure upon injection of the anticancer peptides at the air-water interface which indicates the surface activity of the designed anticancer peptides. Increasing the concentration of the peptide in the subphase resulted in higher surface pressure indicating that the interfacial adsorption of the peptides is concentration dependent.

The surface pressure of the peptides was directly proportional to their β-sheet content in SDS micelles (**Figure 4B**) and inversely proportional to their hydrophobicity (**Figure 4C**). This observation suggests the same conformational changes that happen at the oil-water interface of the SDS micelles may also take place upon adsorption of the peptides at the air-water interface to allow for insertion of the polar surface of the β-sheet into the subphase and projection of the hydrophobic surface of the β-sheet outside the water in the air to achieve the most stable (lowest energy) status. A conformational change from random coil to β-sheet structure has been reported by Maget-Dana et al [81] for a cationic amphiphilic peptide consisting of Leucine and Lysine residues, $(LK)_{50}$, upon

forming a peptide monolayer on the surface of water [70, 81]. The adverse effect of increased hydrophobicity on the surface activity suggests the importance of the hydrophobic-hydrophilic balance (i.e., amphiphilicity) for the optimal surface activity of the peptides. Thus, the surface activity of the cationic amphiphilic peptides is directed by an interplay between their hydrophobicity and their secondary structure.

There was a direct relationship between the surface pressure of the peptides and their anticancer activity (**Figure 4D-E**) while the toxicity of the peptides in HDFs was inversely proportional to their surface activity (**Figure 4F**). These findings suggest that the surface activity of the peptides has a determining role in their anticancer activity and toxicity to normal cells. Although there is no literature data on the surface activity of the β-sheet forming ACPs, the influence of the surface activity of the peptides on their antimicrobial or anticancer activity has been reported for some α-helical AMPs and ACPs [40, 41, 62], in which the biological activity was a function of the surface activity. Hence, measuring the surface activity can provide a tool for predicting the biological activity of this class of cationic amphiphilic peptides and modifying the peptide design accordingly to achieve higher anticancer activity.

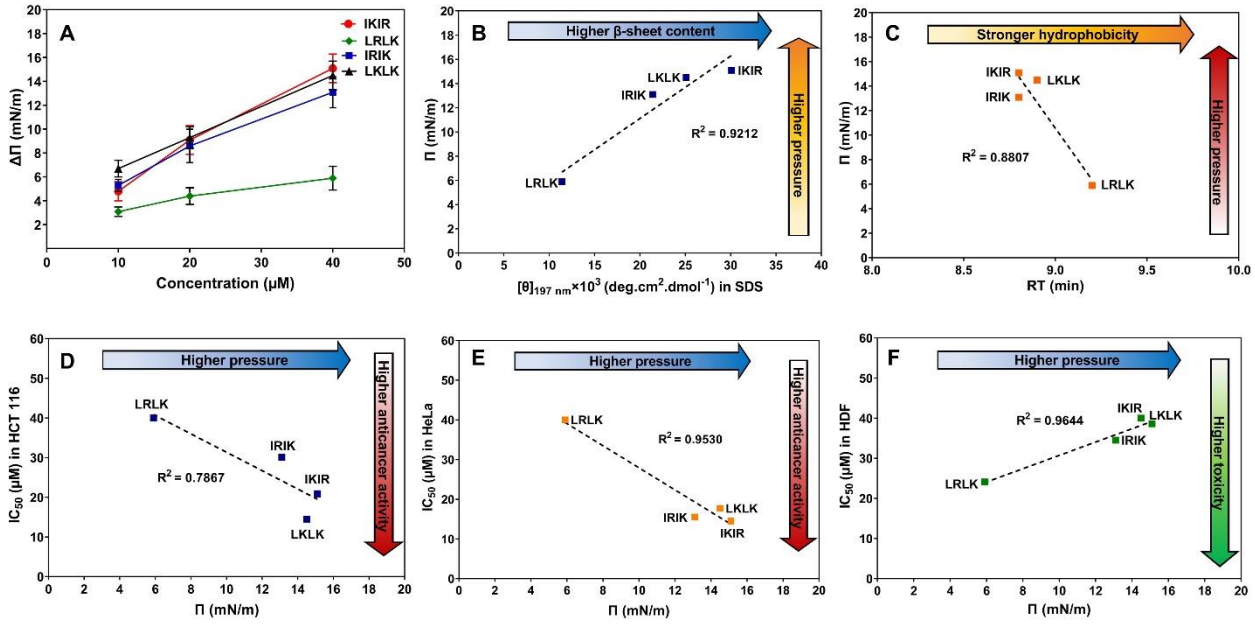


Figure 4. Increase in surface pressure at the air-water interface following injection of different concentrations of the anticancer peptides (**A**). Correlation between the surface pressure of the cationic amphiphilic peptides and their β-sheet content in SDS micelles **(B).** Correlation between the surface pressure of the cationic amphiphilic peptides and their hydrophobicity (**C**). Correlation between the cytotoxicity of the peptides in HCT 116 **(D)**, HeLa **(E),** HDF **(F)** and increase in surface pressure upon adsorption of the peptides (40 μM) at the air-water interface.

Penetration of the anticancer peptides into phospholipid monolayers reveals their mechanism of cell selectivity

As mentioned previously, it is widely accepted that the higher selectivity of the ACPs for cancer cells compared to normal cells is due to their higher tendency for interaction with the anionic cancer

cell membranes compared to the zwitterionic normal cell membranes [32-37, 39]. In order to investigate the interaction of our designed anticancer peptides with different types of cell membranes, negatively charged DPPG lipid monolayers and zwitterionic DPPC lipid monolayers were used as a model for the outer leaflet of the cancer cell and normal cell membrane respectively [61, 71, 82]. The increase in the surface pressure of the lipid monolayer upon injection of the peptide solution in the subphase at a constant surface area is indicative of peptide insertion into the lipid monolayer [61, 62, 71].

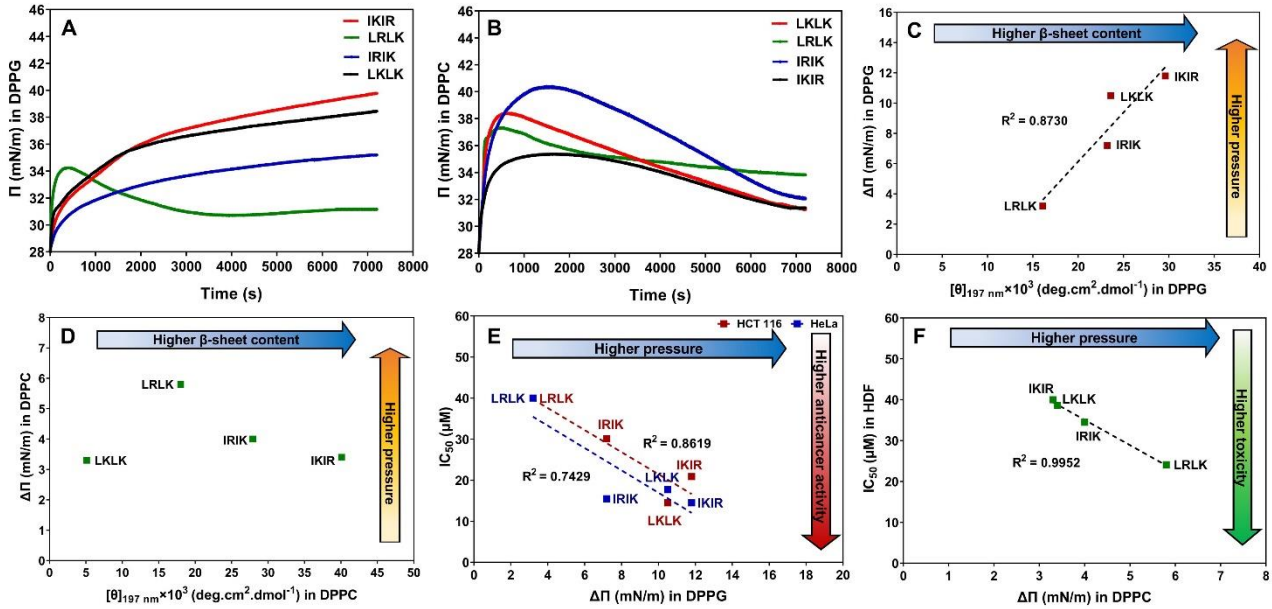


Figure 5. Increase in the pressure of the DPPG **(A)** and DPPC **(B)** lipid monolayers upon injection of the anticancer peptides (20 μM) in the subphase. Correlation between the β-sheet content of the peptides in DPPG SUVs and increase in the surface pressure of DPPG monolayers **(C).** Correlation between the β-sheet content of the peptides in DPPC SUVs and increase in the surface pressure of DPPC monolayers **(D)**. Correlation between cytotoxicity of the peptides in cancer cells and increase in the surface pressure of DPPG lipid monolayers **(E)**. Correlation between cytotoxicity of the peptides in HDF and increase in the surface pressure of DPPC lipid monolayer **(F)**.

The changes to the surface pressure following injection of the anticancer peptides under DPPG and DPPC monolayers are presented in **Figure 5A-B**. Three peptides with highest anticancer activity and highest selectivity for cancer cells (IKIR, IRIK, and LKLK) and one peptide with poor anticancer activity and no selectivity for cancer cells (LRLK) were included in these experiments for comparison. As it is evident from these plots, the anticancer peptides which were more selective for cancer cells than fibroblasts (IKIR, IRIK, and LKLK) had considerably higher affinity for DPPG lipid monolayers than DPPC lipid monolayers and penetrated more into DPPG monolayers. On the other hand, LRLK which lacked toxicity against cancer cells and was toxic to fibroblasts penetrated into DPPC lipid monolayers more than DPPG monolayers and more than the other peptides (**Figure 5B**). These data confirm that the higher selectivity of the designed anticancer peptides for cancer cells results from their higher affinity for cancer cell membranes and greater penetration into the cancer cell membranes. Similar results were reported for the β-sheet forming cell penetrating peptide M918

which exhibited higher affinity for the anionic DOPG lipid monolayer compared to the zwitterionic DOPC lipid monolayer [83]. Also the linear form of the antimicrobial peptide arenicin showed higher affinity for DPPG lipid monolayers than DPPC lipid monolayers whereas its cyclic analogue which was highly toxic to human erythrocytes showed higher affinity for DPPC monolayers [53]. These findings suggest the lipid monolayers as a suitable *in vitro* model for studying the interaction of the anticancer peptides with the cell membranes and predicting their cytotoxicity against normal and cancer cells. The suggested biophysical method could be used as powerful tool for pre-screening the newly designed anticancer peptides prior to performing the costly cell-based screening.

The increase in surface pressure of DPPG monolayers following insertion of the peptides was well correlated to the β-sheet content of the peptides in DPPG SUVs (**Figure 5C**) suggesting that the same conformational changes from random coil to β-sheet that occur upon interaction of the peptides with the lipid bilayer of DPPG SUVs may also occur upon interaction of the peptides with the DPPG lipid monolayers. Conformational change from random coil to β-sheet upon interaction with DPPG monolayers has been previously demonstrated for the linear analogue of arenicin using Infrared reflection absorption spectroscopy (IRRAS) [53]. It is assumed that the electrostatic interaction between the positively charged arginine and lysine residues of the peptides and the negatively charged headgroups of the phospholipids provides initial binding of the peptide to the surface of the lipid monolayer. Subsequently, the peptide transforms from random coil structure into β-sheet structure with separate hydrophobic and hydrophilic surfaces. This conformational change allows for hydrophobic interactions between the isoleucine and leucine residues in the hydrophobic surface of the peptide and the acyl chains of the phospholipids which disturbs the acyl chain ordering of the lipid monolayer manifested by an increase in surface pressure. The suggested molecular mechanism of peptide penetration into lipid monolayers has also been reported for other types of lysine and leucine-based peptides [81, 84]. On the other hand, lack of good correlation between the secondary structure in DPPC SUVs and the pressure increase in DPPC lipid monolayers (**Figure 5D**) suggests that penetration of the designed ACPs into zwitterionic DPPC monolayers is mainly a result of hydrophobic interactions between the hydrophobic amino acid residues of the peptide and the acyl chains of DPPC.

There was a good correlation between the anticancer activity of the peptides in HCT 116 and HeLa and the increase in surface pressure of the DPPG monolayers induced by them (**Figure 5E**). In a similar fashion, the increase in surface pressure of DPPC monolayers upon injection of the anticancer peptides was strongly correlated to their toxicity in HDF cells (**Figure 5F**). These data further confirm that the selectivity of the anticancer peptides for cancer cells results from higher penetration into the cancer cell membranes compared to normal cell membranes presumably due to the difference in their surface charge.

The anticancer peptides exert their cytotoxicity by damaging the mitochondria

Interaction of the designed anticancer peptides with the mitochondrial membranes was investigated by assessing the mitochondrial membrane depolarization following treatment with the peptides. The high content images of HCT 116 and HDF cells stained with JC-1 after treatment with the anticancer peptides LKLK, IKIR, and IRIK are presented in **Figure 6**. These three peptides were selected for this experiment due to their higher selectivity for cancer cells compared to fibroblasts. JC-1 is a mitochondrial probe and its accumulation in the mitochondria depends only on the mitochondrial membrane potential [85, 86]. While the accumulation of JC-1 in healthy mitochondria gives rise to red fluorescence, depolarization of the mitochondrial membranes in pre-apoptotic cells results in reduced accumulation of JC-1 indicated by a shift from red to green fluorescence [87, 88]. Hence, the higher proportion of green to red fluorescence observed in HCT 116 after treatment with the anticancer peptides denotes damage to the mitochondrial membrane whereas higher red to green fluorescence ratio in HDF cells indicates less damage to the mitochondria. This is consistent with the literature data reporting mitochondrial membrane disruption as one of the common mechanisms of action for anticancer peptides [32, 34, 35, 37, 39]. The high affinity of the anticancer peptides for mitochondrial membrane is attributed to its high content of anionic lipids such as cardiolipin [32, 34, 35, 37, 39].

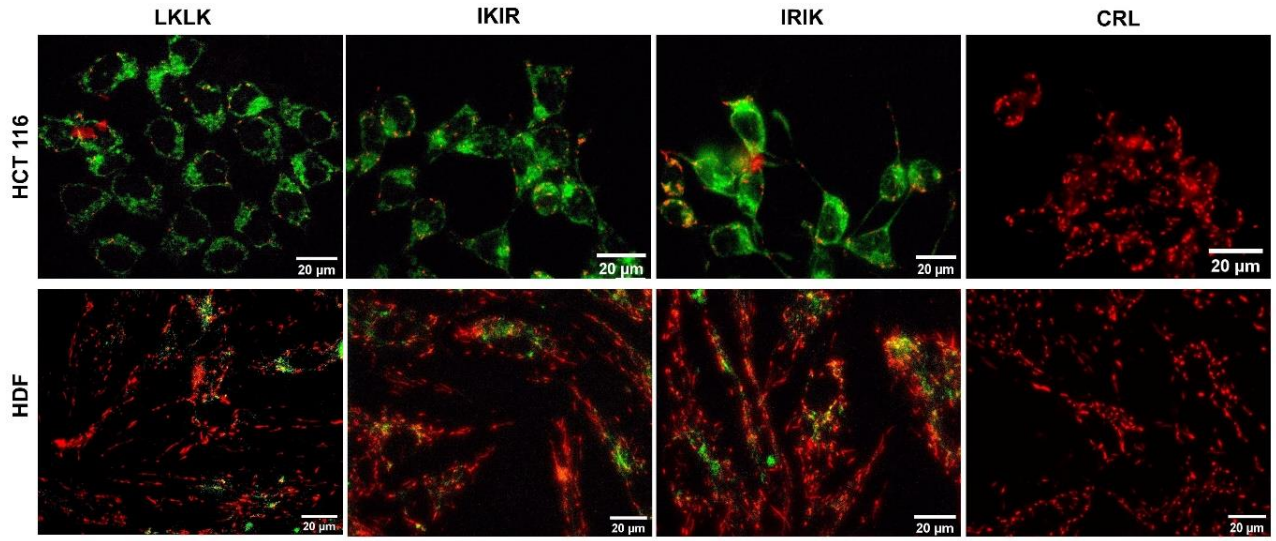


Figure 6. Mitochondrial membrane depolarization by the anticancer peptides in HCT 116 (**top panel**) and HDF cells (**bottom panel**) as determined by JC-1 mitochondrial probe. The red fluorescence indicates healthy mitochondria, and the green fluorescence indicates damaged mitochondria in pre-apoptotic cells.

Conclusion

Herein, we report the development of a new series of β-sheet forming anticancer peptides and their structure activity relationship. The secondary structure of the peptides before and after interaction with model membranes was studied by CD spectroscopy and their surface activity at the

air-water interface as well as their penetration into model lipid monolayers was studied using Langmuir-tensiometer. Some of our designed peptides had higher toxicity to cancer cells compared to fibroblasts and some had a stronger anticancer effect against HeLa cervical cancer cells compared to HCT 116 colorectal cancer cells. It has been found that the best combination of amino acids for achieving high anticancer activity against HCT 116 is the combination of leucine and lysine (LKLK) whereas in HeLa cells the most toxic peptide was IKIK although IKIR, IRIK, and LKLK were all very toxic to HeLa. These observations suggest that in addition to general toxicity to cancer cells, this class of peptides possess some cancer cell specific cytotoxicity. On the other hand, the peptides consisting of leucine in their hydrophobic domain and arginine in their hydrophilic domain (LRLR, LRLK, and LKRL) had considerable toxicity against fibroblast cells and which is not favourable. Hence, this combination of amino acids is not recommended for the β-sheet forming cationic amphiphilic anticancer peptides. The anticancer activity of the peptides was found to be strongly correlated with their secondary structure, their amphiphilicity and surface activity. This has also been reported for other types of short cationic amphiphilic antimicrobial and anticancer peptides with α-helical structure [40, 41, 61, 62, 89]. The higher selectivity of the peptides for cancer cells was found to be a result of their higher affinity for the negatively charged membranes compared to zwitterionic membranes as revealed by higher penetration of the peptides into negatively charged lipid monolayers. This has been generally accepted as one of the main mechanisms of cell selectivity for anticancer peptides [32-37, 39]. The peptides exerted their anticancer activity by damaging the mitochondrial membranes leading to apoptosis, which has been widely reported as one of the main mechanisms of cytotoxicity for many anticancer peptides [32, 34, 35, 37, 39]. The results from this study serve as a guide for the structure-activity relationship of this class of anticancer peptides and will contribute to the development of anticancer peptides with enhanced efficacy and selectivity. Future work is mainly directed towards improvement of the structural design for this type of peptides to enhance their anticancer activity and broaden their anticancer spectrum. Investigating the peptide aggregation into higher ordered structures in membrane environments, the structural features of such aggregates and the exact mechanism of cell membrane disruption by the peptides (i.e., carpet, barrel and stove, toroidal pore, etc.) is also another area to be covered by future studies.

CRediT authorship contributions statement

RH: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Data curation, Visualization, Writing - Original Draft; MAT: Methodology, Writing - Review & Editing; SB: Supervision; JW: Resources; XZ: Conceptualization, Methodology, Visualization, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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