Conjugation of proapoptotic peptides with folate and cyanine dyes for enhanced potency and selectivity towards tumor cell lines

Davide Cardella, Louis Y. P. Luk and Yu-Hsuan Tsai**

ABSTRACT: Despite continuous advances, anticancer therapy still faces several technical hurdles such as selectivity on cellular and subcellular targets of therapeutics. Toward addressing these limitations, we have combined the use of proapoptotic peptides, cyanine dyes and folate to target the mitochondria of tumor cells. Hence, a series of proapoptotic peptides and their conjugates with a cyanine dye and/or a folate were prepared via solid-phase peptide synthesis (SPPS) and their activity tested in different mammalian cell lines. Compounds bearing either a cyanine dye or folate were found to be more cytotoxic than the parent peptides or more selective towards cells overexpressing the folate receptor α, which is commonly found on the surface of tumor cells. Nevertheless, constructs containing both components showed diminished potency and selectivity.

Keywords: Cyanine dye, folate, proapoptotic peptides, mitochondria, cancer, solid-phase peptide synthesis, apoptosis.

INTRODUCTION

Cancer is a leading cause of death worldwide.[1] It is estimated that more than 50% of the people in the UK who are currently under the age of 65 will be diagnosed with cancer during their lifetime.[2] While significant research has been carried out in therapeutic development, treatment of many

different cancers still remains as an unmet clinical need.[3, 4] Thus, there is a necessity for new therapeutic approaches.

One way to overcome some limitations of conventional cancer therapy is via the selective delivery of anticancer drugs to specific organelles within the target cancer cells. This strategy can maximize potency and minimize off-target side effects of the drugs.[5, 6] Amongst the cellular organelles, the mitochondrion hosts several putative drug targets of cancer therapy as the organelle plays key functions in different physiological and pathological cellular processes, such as programmed cell death.[7] Hence, mitochondria are an appealing target for cancer therapies.[8] Many heptamethine cyanine dyes have been demonstrated to preferentially localize in mitochondria and employed as imaging probes.[9-12] The mitochondrial targeting ability is believed to be associated with the delocalized lipophilic cationic nature of the molecules. Indeed, we and others have demonstrated depolarization of mitochondrial membrane prevents accumulation of cyanine dyes in mitochondria.[13, 14] Moreover, we recently showed that tri- and pentamethine cyanine dyes can function as delivery vectors selectively targeting mitochondria with moderate preference toward tumor cells.[13] It was therefore intriguing to us to investigate whether the addition of a cell-targeting component could further improve the cell selectivity of the conjugates.

Receptor-mediated uptake has been utilized for cell-specific delivery. This system takes advantage of the overexpression of a specific receptor on the surface of tumor cells for the selective uptake of anticancer cargoes. One of the most exploited receptors for this strategy is the folate receptor α. To date, many anti-cancer therapeutics have been preferentially delivered to tumor cells upon conjugation to folate.[15-19] Some of the most successful examples reported in the literature are the conjugation of folate to a desacetyl vinblastine monohydrazine[20] and to taxol derivatives.[21] In both cases, the drug and the folate component were separated by a peptidyl spacer and a self-cleaving linker. Besides, folate conjugation was also proven to be effective in the delivery of antibodies, nanoparticles, and imaging agents.[16-18] Specifically, folate conjugates were found to have enhanced selectivity towards cancer cells overexpressing folate receptor α.

Here, we combined selective subcellular and receptor-mediated drug delivery strategies with the view of improving the potency and the selectivity of a series of proapoptotic peptides towards mammalian cells (Figure 1a). Cyanine dyes **1** and **2** (Figure 1b) and folate were chemically conjugated to the N-terminus of three proapoptotic peptides (**3**–**5**) to yield conjugates **6**–**15** (Table 1). The cytotoxicity of these constructs was evaluated in cancer (i.e., KB, MCF7) and non-cancer (i.e., HEK293) cell lines.

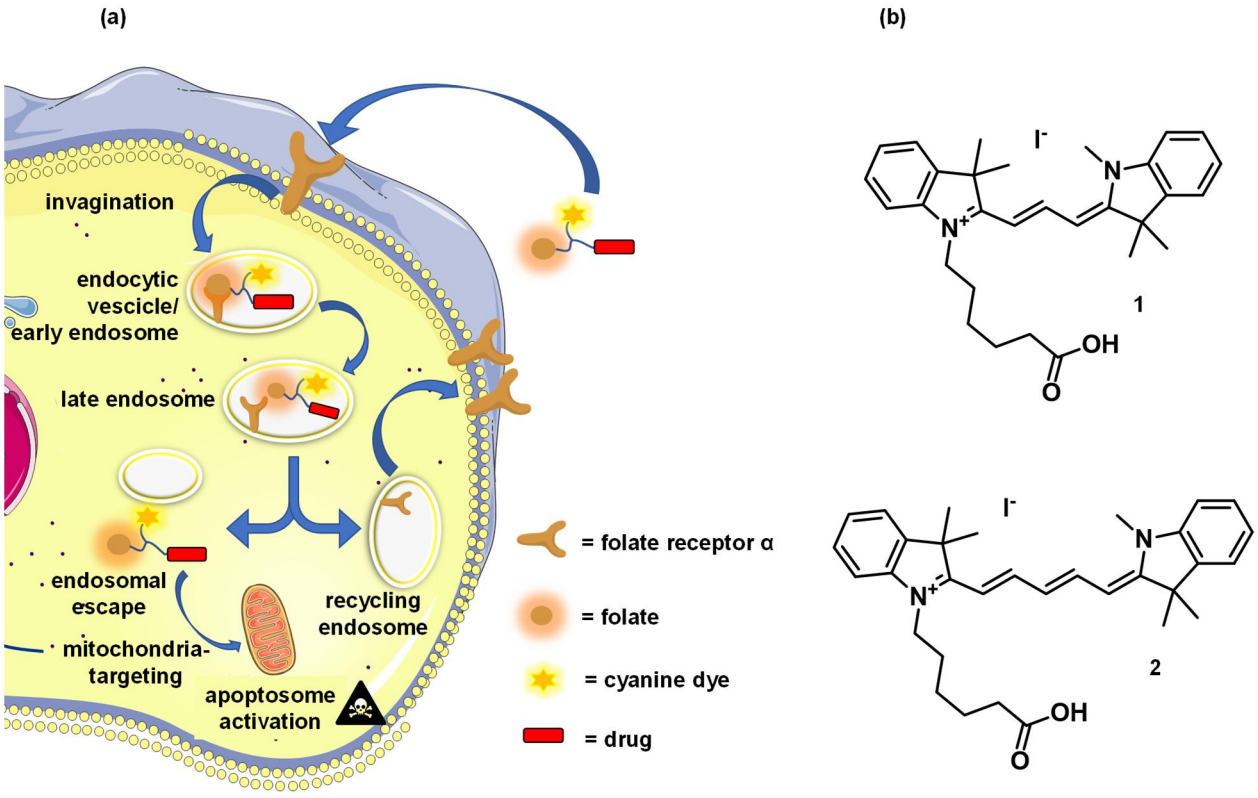
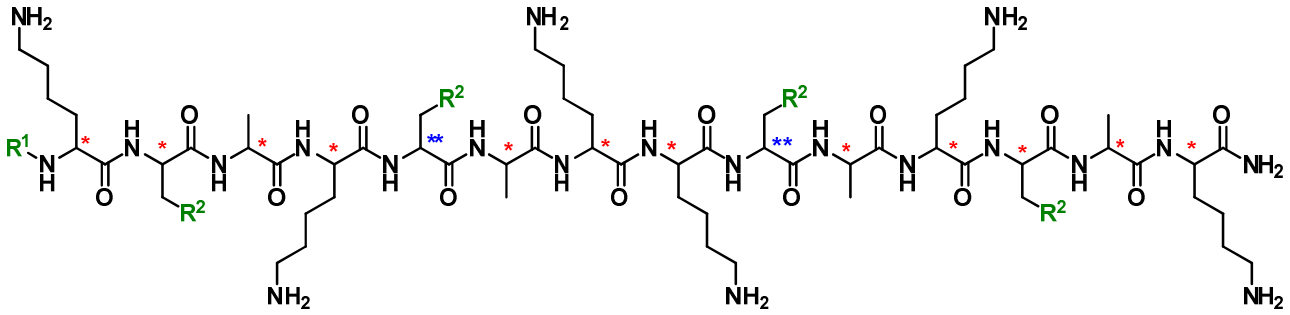
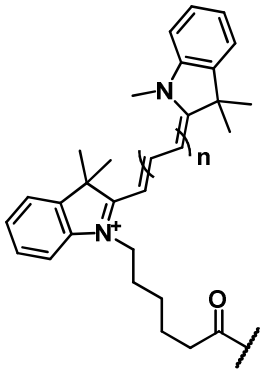


Figure 1. (a) Folate receptor-mediated endocytosis mechanism and cyanine dye-mediated mitochondria-targeting in mammalian cells; (b) Chemical structure of cyanine dyes **1** and **2**.

Table 1. Chemical structure of **3**–**15** and cytotoxicity values for compounds **1**-**15**. The EC_{50} values on different cell lines of different molecules and their cyanine dye conjugates were quantified using

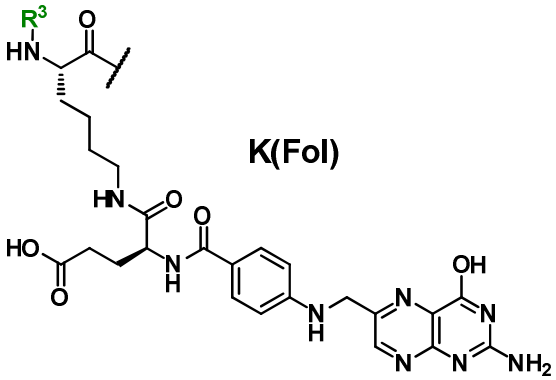
cell viability assays. The values in brackets represent the standard error of the curve fitted using Origin 2019b.





n = 1, Cy3

n = 2, Cy5



						EC_{50} (μM)		
Compound	R^1	R^2	R^3	*	**	KB	MCF7	HEK
1	-	-	-	-	-	36.5 (3.6)	110 (13.2)	221 (53.8)
2	-	-	-	-	-	56.9 (4.1)	59.9 (12.2)	98.8 (21.3)
3	H	isopropyl	-	(*S*)	(*S*)	454 (35.3)	679 (132)	2888 (169)
4	H	isopropyl	-	(*R*)	(*R*)	331 (152)	394 (102)	659 (56.3)
5	H	cyclohexyl	-	(*R*)	(*S*)	6.5 (0.5)	50.9 (3.0)	15.6 (2.6)
6	Cy3	isopropyl	-	(*S*)	(*S*)	6.7 (0.2)	7.37 (0.2)	44.3 (7.0)
7	Cy3	isopropyl	-	(*R*)	(*R*)	3.5 (0.1)	5.7 (0.7)	5.3 (0.2)
8	Cy3	cyclohexyl	-	(*R*)	(*S*)	5.5 (0.5)	11.6 (1.5)	8.2 (0.5)
9	Cy5	isopropyl	-	(*S*)	(*S*)	11.9 (1.2)	15.7 (3.4)	21.3 (6.4)
10	K(Fol)	isopropyl	Ac	(*S*)	(*S*)	242 (33.2)	> 400	> 400

11	K(Fol)	isopropyl	Ac	(*R*)	(*R*)	151 (38.7)	> 400	463 (23.8)
12	K(Fol)	cyclohexyl	Ac	(*R*)	(*S*)	9.8 (0.6)	26.9 (7.2)	20.4 (5.7)
13	K(Fol)	isopropyl	Cy3	(*S*)	(*S*)	38.8 (6.6)	49.4 (4.3)	35.6 (3.4)
14	K(Fol)	isopropyl	Cy3	(*R*)	(*R*)	22.4 (1.4)	13.2 (5.6)	6.9 (1.8)
15	K(Fol)	cyclohexyl	Cy3	(*R*)	(*S*)	3.4 (0.7)	6.9 (0.9)	6.8 (1.4)

* and ** refer to the absolute configuration of the chiral center; Ac = acetyl; pairing anion for compounds **6**–**9** and **13**–**15** is assumed to be trifluoroacetate.

Results

Synthesis of peptides and peptide conjugates **3**–**15**

The chemical synthesis of **3**–**15** was achieved on a polystyrene-based Rink amide resin. Peptides **3**–**5** were synthesized on an automated microwave peptide synthesizer. Initial attempts using N,N′-diisopropylcarbodiimide (DIC)/oxyma coupling strategy and 10% (w/v) piperazine in ethanol:NMP (1:9) as the deprotection cocktail yielded both the desired peptide and a truncated side product that lacks an N-terminal lysine residue (Supplementary Figure S1). This issue was addressed by extending the incubation time of the N-terminal lysine coupling (see Experimental section). After the synthesis on the solid support, the peptide was cleaved by treatment with trifluoroacetic acid, precipitated in cold diethyl ether, purified by reversed-phase HPLC (RP-HPLC) and lyophilized.

Peptidyl backbones of conjugates **6**–**9** were synthesized following the optimized protocol for the synthesis of peptides **3**–**5**. After coupling and deprotection of the last amino acid residue, the peptidyl resin was manually coupled to three equivalents of cyanine dye **1** (for compounds **6**–**8**) or **2** (for compound **9**) in a polypropylene tube at 40 °C for overnight incubation. The desired conjugates were obtained after global deprotection, purification, and lyophilization.

For the synthesis of folate-containing conjugates **10**–**15**, an extra lysine was added at the N-terminal of the peptidyl backbone, and cyanine dye **1** and folate were coupled to its α- and ε-amine groups, respectively. A literature procedure was first attempted.[22] However, this strategy led to folate conjugation at both its α- and γ-carboxylate groups, giving an inseparable mixture of labeled isomers

(Supplementary Figure S2). To overcome this issue, a protected glutamic acid residue, Fmoc-Glu(OtBu)-OH, was first coupled to the peptide, followed by coupling with pteroic acid. Since no difference was found in the endocytosis efficiency of conjugates where the folate is labelled through either its α- or γ-carboxylic group,[23] we opted for the reaction of the α-carboxylate due to the ready availability of the reagent. As a protecting group for the extra lysine side chain to be conjugated with folate, 4-methyltrityl (Mtt) was chosen for conjugates **10**–**14**. In this way, the protecting group on the N-terminal lysine could be selectively cleaved with conditions orthogonal to the other protecting groups on the peptidyl resin. However, the synthesis of compound **15** proved to be more challenging. In fact, upon glutamic acid coupling and Fmoc deprotection, the Mtt strategy led to the presence of a peak in the LC-MS chromatogram with a difference of +129 m/z compared to the desired intermediate, attributable to an extra glutamic acid residue (Supplementary Figure S3). This is likely due to Boc deprotection during the Mtt cleavage conditions (1% TFA in DCM). Finally, conjugate **15** was obtained using 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde) as a protecting group, which could be cleaved in 4% (v/v) hydrazine hydrate in DMF, leading to selective labelling of one folate molecule to the peptide.

Cytotoxicity of **1**–**15**

Cytotoxicity of **1**–**15** was evaluated via CellTiter-Blue assay on KB, MCF7 and HEK293 cells. KB cells are derived from cervical cancer[24] with high-levels of folate receptor α on the cell surface.[17] This feature is of particular relevance in anticancer applications, as it can be exploited for selective targeting and delivery. MCF7 is a cell line derived from human breast cancer with low-levels of folate receptor α.[25] Therefore, these cells can serve as the negative control for folate receptor α mediated tumor-targeted drug delivery systems. HEK293 is a non-cancer cell line and was chosen to evaluate the specificity of the tested compounds towards cancer cells. Cell viability assay results are summarized in Table 1.

Peptides **3** and **4** were found to be the least potent amongst the tested compounds, whereas peptide **5** showed good potency in all tested cell lines. In fact, peptide **5** was designed by engineering the sequence of compound **3** and **4** via replacement of the leucine with cyclohexylalanine residues, which led to an increased cytotoxicity.[26] Cyanine dye-labelled constructs **6**–**9** were found to be significantly more potent ($p < 0.0001$) than the peptides alone (**3**–**5**) in the tested cell lines - the only exception being the cytotoxicity of compound **8** in KB cells, which was not found to be significantly different ($p = 0.11$) to that of its native sequence. Conjugates **10** and **11** bearing a folate component showed enhanced toxicity in KB cells when compared to MCF7 and HEK293 cells. Likewise, cytotoxicity of compound **12** in KB cells was found to be significantly higher ($p < 0.0001$) than in the other tested cell lines. Nevertheless, potency of **10**–**11** in cells not overexpressing folate receptor α is comparable to that of their native peptides. Lastly, dual labelled conjugates **13**–**15** showed enhanced potency compared to the parent sequences and to compounds **10**–**12** ($p < 0.05$), although no selectivity was observed toward KB cells.

Discussion and conclusions

Both organelle-specific and receptor-mediated drug delivery systems have proven to be promising tools in anticancer therapy. We have previously shown the efficacy of tri- and pentamethine cyanine dyes in selectively delivering different cargos to the mitochondria of human cancer cell lines.[13] Many cancer cells overexpress the folate receptor α,[17] enabling the use of folate for targeting cancer cells, especially those derived from ovarian, breast and lung carcinomas.[16] With these premises, we sought to improve the efficacy of existing proapoptotic peptides via conjugation with either cyanine dyes (compounds **6**–**9**) or folate (**10**–**12**). We also attempted to combine the subcellular and receptor mediated delivery strategies by labelling those sequences with both cyanine dye **1** and folate (**13**–**15**). The syntheses were accomplished with as little as two equivalents of Fmoc-protected amino acid per coupling, whereas literature procedures for preparing peptides **3** and **4** employed four or more

equivalents.[22, 27] This is of great relevance when using more expensive D-amino acid or unnatural amino acid (e.g., cyclohexylalanine) building blocks.

Cytotoxicity results, obtained by the CellTiter-Blue assay, are shown in Table 1. The low activity of peptides **3** and **4** in the tested cell lines (EC_{50} > 300 μM) is likely due to their low cell permeability.[27-33] In contrast, peptide **5** was engineered to be more hydrophobic than compounds **3** and **4** via a leucine to cyclohexylalanine mutation; this modification likely led to an increased membrane permeability.[26] Indeed, the cytotoxicity data of **5** obtained here is comparable to the reported values,[26] reinforcing the idea that increased hydrophobicity may lead to a better mitochondrial targeting peptide.[26, 34, 35]

Delocalized lipophilic cations, such as triphenylphosphonium group and the cyanine dyes used here, preferentially localize within mitochondria. While triphenylphosphonium group is widely used to generate mitochondrial-targeting entities,[22] conjugation of peptide **3** triphenylphosphonium group showed negligible toxicity in mammalian cells. In contrast, cyanine dye-labelled constructs **6**–**9** were all found to be more potent than the peptides alone (**3**–**5**) in the tested cell lines. The enhanced potency of these constructs may be due to the increased mitochondrial targeting ability and membrane permeability upon conjugation with cyanine dyes **1** and **2**.

Compounds **10**–**12**, which bear a folate component on the side chain of the N-terminal lysine, show a potency comparable to the parent sequences **3**–**5** towards MCF7 and HEK293 cells. Nevertheless, those constructs all show increased selectivity for KB cells, as envisioned.

Intrigued by the positive effect in terms of potency displayed by cyanine dye conjugation and of selectivity showed by folate labelling, we sought to combine both strategies and generate dual labelled constructs **13**–**15**, where the α-amine of the N-terminal lysine is conjugated to cyanine dye **1** and the side chain of the same lysine is conjugated to the folate. Notwithstanding the enhanced potency compared to the parent sequences and to compounds **10**–**12**, dual labelled constructs did not display the expected selectivity towards KB cells. The observed lack of selectivity may be due to a failed interaction between the folate component and the folate receptor α because of the presence of the

cyanine dye on the same residue. In fact, the steric environment around the folate fragment is known to be an important factor for the interaction of the conjugate with the receptor.[23] Therefore, future work will vary folate and cyanine dye position in the construct to obtain dual labelled compounds with the best cytotoxicity and selectivity.

In conclusion, cyanine dyes and folate are promising tools to further improve potency and selectivity of drugs in anticancer therapy. Nevertheless, further effort must be made to optimize the design of constructs bearing both components.

Experimental section

General Information

SPPS was performed on a CEM Liberty Blue™ Automated Microwave Peptide Synthesizer and in TELOS Filtration Columns comprise equipped with 20 µm polyethylene frits. Reaction temperatures are stated as heating device temperature of the synthesizer, if not otherwise stated. Deionized water was obtained by an Elga PURELAB 8 Option system (15 MΩ·cm). Reagents obtained from commercial suppliers were used without further purification unless otherwise stated. Protected amino acids and triisopropylsilane (TIS) were purchased from Cambridge Reagents. Rink Amide 4-methylbenzhydrylamine (MBHA) resin, DIC, oxyma pure, piperazine and trifluoroacetic acid were purchased from Fluorochem. DMF was purchased from Fisher Scientific. Pteroic acid was purchased from Alfa Aesar. For HPLC mobile phase, HPLC grade CH_3CN from Fisher Scientific and trifluoroacetic acid from Fluorochem were used. All were laboratory reagent grade and were used without further purification unless otherwise noted. LC-MS was performed on a 1260 Infinity II from Agilent Technologies. Semi-preparative RP-HPLC was performed on a 1206 Infinity from Agilent Technologies equipped with a Phenomenex Gemini C18, 10.0 x 250 mm, particle size 10 µm, pore size 110 Å or with an ACE C18, 10.0 x 250 mm, particle size 5 µm, pore size 100 Å. Determination of Rink Amide MBHA resin loading was conducted using a ThermoFisher NanoDrop ND-ONE-W

spectrophotometer. Absorbance values were measured in 10 mm path-length cuvettes (Fisher Scientific, #11847832).

Determination of Rink Amide resin loading

10 mg of resin was weighed into an Eppendorf tube, to which 800 µL of DMF was added. After allowing the resin to swell for 15 min, 200 µL of piperidine was added to the suspension; at this point the tube was vortexed and allowed to stand for 15 min at room temperature. Afterwards, the tube was centrifuged and 100 µL of the supernatant transferred to a 1 cm path-length cuvette and diluted with 900 µL of DMF. The absorbance at 301 nm was measured versus a blank, which was prepared with the same procedure but without the use of the resin. The concentration, c, of dibenzofulvene-piperidine adduct released from the resin was determined by applying the Lambert-Beer law [$A= \varepsilon c l$], where A is the absorbance value, l is the path length (1 cm) and ε is the extinction coefficient of the dibenzofulvene-piperidine adduct (7800 mL/mmol*cm, λ = 301 nm).[36] Average values resulted from three independent experiments. Finally, the loading, L, was calculated using the formula:

$L = c \times V \times d / m$

Where V is the volume of solution in the cuvette, d is the dilution coefficient, and m is the mass of the weighted resin. In the described procedure, V = 1 mL, d = 10 and m = 10 mg.

Chemical Synthesis

Synthesis of cyanine dye scaffolds **1** and **2**

Compounds **1** and **2** were prepared using the literature procedure.[37]

SPPS of compounds **3-5**

The peptide chain was synthesized by SPPS on Rink Amide MBHA resin. Each amino acid coupling step was carried out with Fmoc-protected amino acid (2 eq), DIC (4 eq), ethyl cyanohydroxyiminoacetate (2 eq) in DMF at 75 °C (155 W) for 0.25 min and at 90 °C (30 W) for further 2 min. For the last lysine, the coupling was carried out at 75 °C (155 W) for 0.5 min and at 90 °C (30 W) for further 4 min. After each coupling step, the respective Fmoc protecting group was removed by 10% Piperazine (w/v) in ethanol:NMP (1:9). After the final Fmoc deprotection, cleavage

was performed in TFA/H_2O/TIS 95:2.5:2.5 solution for 2 h. The resin was removed by filtration and the filtrate was concentrated by N_2 flow. The crude product was precipitated with cold diethyl ether, centrifuged (4,000 g for 10 min) and the supernatant was discarded. The precipitate was dissolved in water, filtrated through a 0.22 µm syringe filter and purified by RP-HPLC. Lyophilization of the pure product fractions afforded the desired compound as white powder, which was characterized by LC-MS (Supplementary Figure S3-S5).

SPPS of compounds **6-9**

The peptide chain was synthesized by SPPS on Rink Amide MBHA resin. Each amino acid coupling step was carried out with Fmoc-protected amino acid (2 eq), DIC (4 eq), ethyl cyanohydroxyiminoacetate (2 eq) in DMF at 75 °C (155 W) for 0.25 min and at 90 °C (30 W) for further 2 min. For the last lysine, the coupling was carried out at 75 °C (155 W) for 0.5 min and at 90 °C (30 W) for a further 4 min. After each coupling step, the respective Fmoc protecting group was removed by 10% Piperazine (w/v) in ethanol:NMP (1:9). After the final Fmoc deprotection, the cyanine dye scaffold was manually coupled using 3 eq of compound **1** or **2**, 6 eq of DIC, 3 eq of ethyl cyanohydroxyiminoacetate for 16 h at room temperature. Cleavage was performed in TFA/H_2O/TIS 95:2.5:2.5 solution for 2 h. The resin was removed by filtration and the filtrate was concentrated by N_2 flow. The crude product was precipitated with cold diethyl ether, centrifuged (4,000 g for 10 min) and the supernatant was discarded. The precipitate was dissolved in water, filtrated through a 0.22 µm syringe filter and purified by RP-HPLC. Lyophilization of the pure product fractions afforded the desired compound as red powder in case of labelling with compound **1**, or blue powder in case of labelling with compound **2**, which were characterized by LC-MS (Supplementary Figure S6-S9).

SPPS of compounds **10-12**

The peptide chain was synthesized by SPPS on Rink Amide MBHA resin. Each amino acid coupling step was carried out with Fmoc-protected amino acid (2 eq), DIC (4 eq), ethyl cyanohydroxyiminoacetate (2 eq) in DMF at 75 °C (155 W) for 0.25 min and at 90 °C (30 W) for further 2 min. For the penultimate lysine, the coupling was carried out using Fmoc-Lys(Boc)-OH (2

eq), DIC (4 eq), ethyl cyanohydroxyiminoacetate (2 eq) at 75 °C (155 W) for 0.5 min and at 90 °C (30 W) for further 4 min. For the last lysine, the coupling was carried out using Fmoc-Lys(Mtt)-OH (3 eq), DIC (6 eq), ethyl cyanohydroxyiminoacetate (3 eq) at 75 °C (155 W) for 1 min and at 90 °C (30 W) for further 7 min. After each coupling step, the respective Fmoc protecting group was removed by 10% Piperazine (w/v) in ethanol:NMP (1:9). After the final Fmoc deprotection, the peptidyl-resin was removed from the automated synthesizer and the N-terminal amine capped using acetic anhydride (50 eq) and pyridine (50 eq) in 8 mL of DMF, for 30 min for 2 times. For the Mtt group cleavage, the resin was treated with 1% TFA (v/v) in DCM for 2 min and then the solution filtrated off; this procedure was repeated 12 times. Then, glutamic acid was coupled using 4 eq of Fmoc-Glu(OtBu)-OH, 4 eq of (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 4 eq of hydroxybenzotriazole (HOBt) and 8 eq of N,N-diisopropylethylamine (DIPEA). Deprotection of the Fmoc protecting group was achieved by treatment with 5 mL of 20% (v/v) piperidine in DMF, for 10 min for 2 times. For the pteroic acid coupling, 2 eq of pteroic acid were suspended in 10 mL of DMSO and heated at 50 °C for 1 h. 8 eq of DIPEA and 2 eq of benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) were added to the suspension which was then reacted with the peptidyl-resin for 16 h, at 40 °C. The pteroic acid coupling was then repeated for a further 16 h, at 40 °C. Cleavage from the resin was performed in TFA/H_2O/TIS 95:2.5:2.5 solution for 2 h. The resin was removed by filtration and the filtrate concentrated by N_2 flow. The crude product was precipitated with cold diethyl ether, centrifuged (4,000 g for 10 min) and the supernatant discarded. The precipitate was dissolved in 2 mL of CH_3CN + 0.1% TFA, diluted with 13 mL of H_2O + 0.1% TFA, filtrated through a 0.22 µm syringe filter and purified by RP-HPLC. Lyophilization of the pure product fractions afforded the desired compound as a pale-yellow powder, which was characterized by LC-MS (Supplementary Figure S10-S12).

SPPS of compounds **13** and **14**

The peptide chain was synthesized by SPPS on Rink Amide MBHA resin. Each amino acid coupling step was carried out with Fmoc-protected amino acid (2 eq), DIC (4 eq), ethyl

cyanohydroxyiminoacetate (2 eq) in DMF at 75 °C (155 W) for 0.25 min and at 90 °C (30 W) for further 2 min. For the penultimate lysine, the coupling was carried out using Fmoc-Lys(Boc)-OH (2 eq), DIC (4 eq), ethyl cyanohydroxyiminoacetate (2 eq) at 75 °C (155 W) for 0.5 min and at 90 °C (30 W) for further 4 min. For the last lysine, the coupling was carried out using Fmoc-Lys(Mtt)-OH (3 eq), DIC (6 eq), ethyl cyanohydroxyiminoacetate (3 eq) at 75 °C (155 W) for 1 min and at 90 °C (30 W) for further 7 min. After each coupling step, the respective Fmoc protecting group was removed by 10% piperazine (w/v) in ethanol:NMP (1:9). After the final Fmoc deprotection, the peptidyl resin was removed from the automated synthesizer and the trimethine cyanine dye was manually coupled using 3 eq of compound **1**, 6 eq of DIC, 3 eq of ethyl cyanohydroxyiminoacetate, 16 h. For the Mtt group cleavage, the resin was treated with 1% TFA (v/v) in DCM for 2 min and then the solution filtrated off. The procedure was repeated 12 times. Then, glutamic acid was coupled using 4 eq of Fmoc-Glu(OtBu)-OH, 4 eq of HBTU, 4 eq of HOBt and 8 eq of DIPEA. Deprotection of the Fmoc protecting group was achieved by treatment with 5 mL of 20% (v/v) piperidine in DMF, for 10 min for 2 times. For the pteroic acid coupling, 2 eq of pteroic acid were suspended in 10 mL of DMSO and were heated at 50 °C for 1 h. 8 eq of DIPEA and 2 eq of Pybop were added to the suspension which was then reacted with the peptidyl-resin for 16 h at 40 °C. The pteroic acid coupling was then repeated for further 16 h at 40 °C. Cleavage from the resin was performed in TFA/H_2O/TIS 95:2.5:2.5 solution for 2 h. The resin was removed by filtration and the filtrate concentrated by N_2 flow. The crude product was precipitated with cold diethyl ether, centrifuged (4,000 g for 10 min) and the supernatant discarded. The precipitate was dissolved in 2 mL of CH_3CN + 0.1% TFA, diluted with 13 mL of H_2O + 0.1% TFA, filtrated through a 0.22 μm syringe filter and purified by RP-HPLC. Lyophilization of the pure product fractions afforded the desired compound as a red powder, which was characterized by LC-MS (Supplementary Figure S13-S14).

SPPS of compound **15**

The peptide chain was synthesized by SPPS on Rink Amide MBHA resin. Each amino acid coupling step was carried out with Fmoc-protected amino acid (2 eq), DIC (4 eq), ethyl

cyanohydroxyiminoacetate (2 eq) in DMF at 75 °C (155 W) for 0.25 min and at 90 °C (30 W) for further 2 min. For the penultimate lysine, the coupling was carried out using Fmoc-Lys(Boc)-OH (2 eq), DIC (4 eq), ethyl cyanohydroxyiminoacetate (2 eq) at 75 °C (155 W) for 0.5 min and at 90 °C (30 W) for further 4 min. For the last lysine, the coupling was carried out using Fmoc-Lys(ivDde)-OH (3 eq), DIC (6 eq), ethyl cyanohydroxyiminoacetate (3 eq) at 75 °C (155 W) for 1 min and at 90 °C (30 W) for further 7 min. After each coupling step, the respective Fmoc protecting group was removed by 10% piperazine (w/v) in ethanol:NMP (1:9). After the final Fmoc deprotection, the peptidyl resin was removed from the automated synthesizer and the trimethine cyanine dye was manually coupled using 3 eq of compound **1**, 6 eq of DIC, 3 eq of ethyl cyanohydroxyiminoacetate, 16 h. For the ivDde group cleavage, the resin was treated with 4% hydrazine hydrate (v/v) in DMF for 45 min and then the solution filtrated off. The procedure was repeated twice. Then, glutamic acid was coupled using 4 eq of Fmoc-Glu(OtBu)-OH, 4 eq of HBTU, 4 eq of HOBt and 8 eq of DIPEA. Deprotection of the Fmoc protecting group was achieved by treatment with 5 mL of 20% (v/v) piperidine in DMF, for 10 min for 2 times. For the pteroic acid coupling, 2 eq of pteroic acid were suspended in 10 mL of DMSO and heated at 50 °C for 1 h. 8 eq of DIPEA and 2 eq of Pybop were added to the suspension, which was then reacted with the peptidyl-resin for 16 h at 40 °C. The pteroic acid coupling was then repeated for further 16 h at 40 °C. Cleavage from the resin was performed in TFA/H_2O/TIS 95:2.5:2.5 solution for 2 h. The resin was removed by filtration and the filtrate concentrated by N_2 flow. The crude product was precipitated with cold diethyl ether, centrifuged (4,000 g for 10 min) and the supernatant was discarded. The precipitate was dissolved in 2 mL of CH_3CN + 0.1% TFA, diluted with 13 mL of H_2O + 0.1% TFA, filtrated through a 0.22 μm syringe filter and purified by RP-HPLC. Lyophilization of the pure product fractions afforded the desired compound as a red powder, which was characterized by LC-MS (Supplementary Figure S15).

Cell Culture

KB and MCF7 were kindly gifted by Prof. Arwyn T. Jones (Cardiff University), and HEK293 cells were purchased from Public Health England. Cells were routinely tested for mycoplasma infection.

KB cells were maintained in T75 flasks at 37 °C in a 5% CO_2 atmosphere in folic acid-depleted Roswell Park Memorial Institute (RPMI)-1640 medium (Fisher Scientific, #11554416) supplemented with 10% (v/v) fetal bovine serum (FBS) (Fisher Scientific, #11573397). MCF7 and HEK293 cells were maintained in T75 flasks at 37 °C in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle's medium (DMEM) (Fisher Scientific, #10566016) supplemented with 10% (v/v) FBS. Cells were maintained at a sub-confluent monolayer and split at 80-85% confluency. For splitting, cells were washed with PBS, trypsinised in 1 mL of trypsin (Fisher Scientific, #11560626) and 200 µL of the 1000 µL trypsin cell suspension was re-suspended in 12 mL fresh DMEM containing 10% (v/v) FBS in a new T75 flask. KB cells were used for biological experiments after being maintained for at least 10 days in folic acid-depleted RPMI-1640 medium.

Cell viability assay

MCF7 and HEK293 cells were seeded at a density of 2×10^4 cells per well in a Nunc™ MicroWell™ 96-well plate (Thermo Scientific, #10174221) and grown at 37 °C in a 5% CO_2 atmosphere in DMEM supplemented with 10% (v/v) FBS for 24 h. KB cells were seeded at a density of 7×10^3 cells per well in a 96-well plate and grown at 37 °C in a 5% CO_2 atmosphere in folic acid-depleted RPMI-1640 medium supplemented with 10% (v/v) FBS for 24 h. Stock solutions of compounds **3**–**7** and **9–11** were obtained by dissolving the compounds in sterile deionized water. Compounds **1**, **2**, **8**, **12–15** were dissolved in pure DMSO. The stock solutions were diluted into the proper medium (according to the cell line tested) supplemented with 10% FBS to the appropriate concentration, and cells in each well were incubated with 100 µL of the solution. The solutions in each well were then adjusted to a concentration of 1% (v/v) DMSO. After 24 h at 37 °C, 20 µL of CellTiter-Blue™ (Promega, #G8080) was added to each well. The plate was incubated for another 4 h at 37 °C before analysis on a Perkin Elmer Victor X plate reader (excitation 531 nm; emission 595 nm). Each data point was calculated from a minimum of nine values resulting from three biological replicates (i.e., cells split from three different passages); each biological replicate is calculated from three technical replicates (i.e., cells split from the same passage). Value from media-only with CellTiter-Blue™ was set as 0% viability.

This value was then subtracted from the values from cell-only (i.e., non-treated) wells with CellTiter-Blue™ in each biological replicate and set as 100% viability. For treatments containing compound **1**, **2** and cyanine dye-labeled constructs, blanks were generated with cell-free wells containing the compounds incubated for 24 h before addition of CellTiter-Blue™. The fluorescent reading for these wells was deducted from the treatment readings.

Statistical analysis

Comparison of cytotoxicity fitting curves was achieved by applying the extra sum-of-squares F test, performed with GraphPad Prism 6. Statistical difference between cytotoxicity values was assumed when *p*-value, as output of the extra sum-of-squares F test, was found to be below 0.05.

Supporting Information

- SPPS procedures for compounds **3–15**; TIC chromatograms of the crude of compound **3** synthesis before and after optimizing the SPPS protocol (Figure S1); TIC chromatogram of the crude for the synthesis of compound **10** after coupling folate to the ε-amino group of the N-terminal lysine. (Figure S2); TIC chromatogram of the crude of synthesis for compound **15** after coupling and deprotection of glutamic acid to the ε-amino group of the N-terminal lysine (Figure S3); characterization data for compounds **3–15** (Table S1 + Figure S4–S16); cytotoxicity data (Figure S17–S19). (PDF)

Author Information

Corresponding Authors

Yu-Hsuan Tsai

School of Chemistry, Cardiff University, Main Building, Park Place, Cardiff, CF10 3AT – UK; https://orcid.org/0000-0003-0589-5088; e-mail: tsai.y-h@outlook.com / tsaiy5@cardiff.ac.uk; Tel.: +86 (755) 2684 9231 / +44-2920-879-285

Louis Y. P. Luk

School of Chemistry, Cardiff University, Main Building, Park Place, Cardiff, CF10 3AT – UK;

Cardiff Catalysis Institute, School of Chemistry, Cardiff University, Main Building, Park Place, Cardiff, CF10 3AT – UK;

https://orcid.org/0000-0002-7864-6261; e-mail: lukly@cardiff.ac.uk; Tel.: +44-29225-10161

Authors

Davide Cardella

School of Chemistry, Cardiff University, Main Building, Park Place, Cardiff, CF10 3AT – UK;

https://orcid.org/0000-0003-1025-8710; e-mail: cardellad@cardiff.ac.uk; Tel.: +44-(0)7446261512

Author Contributions

D.C. performed the synthesis, the characterization, and the biological experiments. D.C., Y.T. and L.L. designed the overall study. D.C wrote the manuscript. Y.T. and L.L. edited the manuscript.

Notes

The authors declare no conflict of interest.

Acknowledgements

We thank Prof. Arwyn T. Jones for the donation of MCF7 cell lines, Dr. Alexander R. Nödling and Dr. Xuefei Li for the help in the organic lab, Emily M. Mills for the help in the cell culture lab and Victoria L. Barlow for language editing and proofreading the manuscript. We are grateful for the financial support from the Welsh Government (Life Sciences Research Network Wales Scholarship to D. C.).

Abbreviations

DIC, N,N′-diisopropylcarbodiimide; DIPEA, N,N-diisopropylethylamine; DMEM, Dulbecco's Modified Eagle's medium; FBS, Fetal bovine serum; HBTU, (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, Hydroxybenzotriazole; ivDde, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl; MBHA, 4-methylbenzhydrylamine; Pybop, Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; RP-HPLC, Reversed-phase

high performance liquid chromatography; RPMI, Roswell Park Memorial Institute; SPPS, Solid-phase peptide synthesis; TIS, Triisopropylsilane.

References

1. Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet* **2016,** *388* (10053), 1459-1544.
2. Torre, L. A.; Siegel, R. L.; Ward, E. M.; Jemal, A., Global Cancer Incidence and Mortality Rates and Trends--An Update. *Cancer Epidemiol. Biomarkers Prev.* **2016,** *25* (1), 16-27.
3. Ahmad, A. S.; Ormiston-Smith, N.; Sasieni, P. D., Trends in the lifetime risk of developing cancer in Great Britain: comparison of risk for those born from 1930 to 1960. *Br. J. Cancer* **2015,** *112* (5), 943-947.
4. Pucci, C.; Martinelli, C.; Ciofani, G., Innovative approaches for cancer treatment: current perspectives and new challenges. *Ecancermedicalscience* **2019,** *13*, 961.
5. Dagogo-Jack, I.; Shaw, A. T., Tumour heterogeneity and resistance to cancer therapies. *Nat. Rev. Clin. Oncol.* **2018,** *15* (2), 81-94.
6. Langer, R., Drugs on Target. *Science* **2001,** *293* (5527), 58-59.
7. Roth, K. G.; Mambetsariev, I.; Kulkarni, P.; Salgia, R., The Mitochondrion as an Emerging Therapeutic Target in Cancer. *Trends Mol. Med.* **2020,** *26* (1), 119-134.
8. Chen, W.-H.; Luo, G.-F.; Zhang, X.-Z., Recent Advances in Subcellular Targeted Cancer Therapy Based on Functional Materials. *Adv. Mater.* **2019,** *31* (3), 1802725.
9. Usama, S. M.; Park, G. K.; Nomura, S.; Baek, Y.; Choi, H. S.; Burgess, K., Role of Albumin in Accumulation and Persistence of Tumor-Seeking Cyanine Dyes. *Bioconj. Chem.* **2020,** *31* (2), 248-259.
10. Yang, X.; Shi, C.; Tong, R.; Qian, W.; Zhau, H. E.; Wang, R.; Zhu, G.; Cheng, J.; Yang, V. W.; Cheng, T.; Henary, M.; Strekowski, L.; Chung, L. W., Near IR heptamethine cyanine dye-mediated cancer imaging. *Clin. Cancer. Res.* **2010,** *16* (10), 2833-2844.
11. Jiang, Z.; Pflug, K.; Usama, S. M.; Kuai, D.; Yan, X.; Sitcheran, R.; Burgess, K., Cyanine–Gemcitabine Conjugates as Targeted Theranostic Agents for Glioblastoma Tumor Cells. *J. Med. Chem.* **2019,** *62* (20), 9236-9245.
12. Liu, Y.; Zhou, J.; Wang, L.; Hu, X.; Liu, X.; Liu, M.; Cao, Z.; Shangguan, D.; Tan, W., A Cyanine Dye to Probe Mitophagy: Simultaneous Detection of Mitochondria and Autolysosomes in Live Cells. *J. Am. Chem. Soc.* **2016,** *138* (38), 12368-12374.
13. Nödling, A. R.; Mills, E. M.; Li, X.; Cardella, D.; Sayers, E. J.; Wu, S.-H.; Jones, A. T.; Luk, L. Y. P.; Tsai, Y.-H., Cyanine dye mediated mitochondrial targeting enhances the anti-cancer activity of small-molecule cargoes. *Chem. Commun.* **2020,** (56), 4672-4675.
14. Onoe, S.; Temma, T.; Shimizu, Y.; Ono, M.; Saji, H., Investigation of cyanine dyes for in vivo optical imaging of altered mitochondrial membrane potential in tumors. *Cancer Medicine* **2014,** *3* (4), 775-786.
15. Leamon, C. P.; Pastan, I.; Low, P. S., Cytotoxicity of folate-Pseudomonas exotoxin conjugates toward tumor cells. Contribution of translocation domain. *J. Biol. Chem.* **1993,** *268* (33), 24847-24854.
16. Cheung, A.; Bax, H. J.; Josephs, D. H.; Ilieva, K. M.; Pellizzari, G.; Opzoomer, J.; Bloomfield, J.; Fittall, M.; Grigoriadis, A.; Figini, M.; Canevari, S.; Spicer, J. F.; Tutt, A. N.; Karagiannis, S. N., Targeting folate receptor alpha for cancer treatment. *Oncotarget* **2016,** *7* (32), 52553-52574.
17. Fernández, M.; Javaid, F.; Chudasama, V., Advances in targeting the folate receptor in the treatment/imaging of cancers. *Chem. Sci.* **2018,** *9* (4), 790-810.

18. Sudimack, J.; Lee, R. J., Targeted drug delivery via the folate receptor. *Adv. Drug Del. Rev.* **2000,** *41* (2), 147-162.
19. Vlahov, I. R.; Leamon, C. P., Engineering Folate–Drug Conjugates to Target Cancer: From Chemistry to Clinic. *Bioconj. Chem.* **2012,** *23* (7), 1357-1369.
20. Leamon, C. P.; Vlahov, I. R.; Reddy, J. A.; Vetzel, M.; Santhapuram, H. K. R.; You, F.; Bloomfield, A.; Dorton, R.; Nelson, M.; Kleindl, P.; Vaughn, J. F.; Westrick, E., Folate–Vinca Alkaloid Conjugates for Cancer Therapy: A Structure–Activity Relationship. *Bioconj. Chem.* **2014,** *25* (3), 560-568.
21. Seitz, J. D.; Vineberg, J. G.; Herlihy, E.; Park, B.; Melief, E.; Ojima, I., Design, synthesis and biological evaluation of a highly-potent and cancer cell selective folate–taxoid conjugate. *Biorg. Med. Chem.* **2015,** *23* (9), 2187-2194.
22. Chen, W.-H.; Xu, X.-D.; Luo, G.-F.; Jia, H.-Z.; Lei, Q.; Cheng, S.-X.; Zhuo, R.-X.; Zhang, X.-Z., Dual-Targeting Pro-apoptotic Peptide for Programmed Cancer Cell Death via Specific Mitochondria Damage. *Sci. Rep.* **2013,** *3* (1), 3468.
23. Leamon, C. P.; Deprince, R. B.; Hendren, R. W., Folate-mediated Drug Delivery: Effect of Alternative Conjugation Chemistry. *J. Drug Target.* **1999,** *7* (3), 157-169.
24. Vaughan, L.; Glänzel, W.; Korch, C.; Capes-Davis, A., Widespread Use of Misidentified Cell Line KB (HeLa): Incorrect Attribution and Its Impact Revealed through Mining the Scientific Literature. *Cancer Res.* **2017,** *77* (11), 2784-2788.
25. Elwood, P. C., Molecular cloning and characterization of the human folate-binding protein cDNA from placenta and malignant tissue culture (KB) cells. *J. Biol. Chem.* **1989,** *264* (25), 14893-14901.
26. Horton, K. L.; Kelley, S. O., Engineered Apoptosis-Inducing Peptides with Enhanced Mitochondrial Localization and Potency. *J. Med. Chem.* **2009,** *52* (10), 3293-3299.
27. Law, B.; Quinti, L.; Choi, Y.; Weissleder, R.; Tung, C. H., A mitochondrial targeted fusion peptide exhibits remarkable cytotoxicity. *Mol. Cancer Ther.* **2006,** *5* (8), 1944-1949.
28. Ellerby, H. M.; Arap, W.; Ellerby, L. M.; Kain, R.; Andrusiak, R.; Rio, G. D.; Krajewski, S.; Lombardo, C. R.; Rao, R.; Ruoslahti, E.; Bredesen, D. E.; Pasqualini, R., Anti-cancer activity of targeted pro-apoptotic peptides. *Nat. Med.* **1999,** *5* (9), 1032-1038.
29. Jung, H. K.; Kim, S.; Park, R. W.; Park, J. Y.; Kim, I. S.; Lee, B., Bladder tumor-targeted delivery of pro-apoptotic peptide for cancer therapy. *J. Control. Release* **2016,** *235*, 259-267.
30. Qiao, Z.-Y.; Lai, W.-J.; Lin, Y.-X.; Li, D.; Nan, X.-H.; Wang, Y.; Wang, H.; Fang, Q.-J., Polymer–KLAK Peptide Conjugates Induce Cancer Cell Death through Synergistic Effects of Mitochondria Damage and Autophagy Blockage. *Bioconj. Chem.* **2017,** *28* (6), 1709-1721.
31. Smolarczyk, R.; Cichoń, T.; Graja, K.; Hucz, J.; Sochanik, A.; Szala, S., Antitumor effect of RGD-4C-GG-D(KLAKLAK)2 peptide in mouse B16(F10) melanoma model. *Acta Biochim. Pol.* **2006,** *53* (4), 801-805.
32. Mai, J. C.; Mi, Z.; Kim, S. H.; Ng, B.; Robbins, P. D., A proapoptotic peptide for the treatment of solid tumors. *Cancer Res.* **2001,** *61* (21), 7709-7712.
33. Kim, H. Y.; Kim, S.; Youn, H.; Chung, J.-K.; Shin, D. H.; Lee, K., The cell penetrating ability of the proapoptotic peptide, KLAKLAKKLAKLAK fused to the N-terminal protein transduction domain of translationally controlled tumor protein, MIIYRDLISH. *Biomaterials* **2011,** *32* (22), 5262-5268.
34. Horton, K. L.; Stewart, K. M.; Fonseca, S. B.; Guo, Q.; Kelley, S. O., Mitochondria-penetrating peptides. *ACS Chem. Biol.* **2008,** *15* (4), 375-382.
35. Horton, K. L.; Pereira, M. P.; Stewart, K. M.; Fonseca, S. B.; Kelley, S. O., Tuning the activity of mitochondria-penetrating peptides for delivery or disruption. *ChemBioChem* **2012,** *13* (3), 476-485.
36. Vergel Galeano, C. F.; Rivera Monroy, Z. J.; Rosas Pérez, J. E.; García Castañeda, J. E., Efficient Synthesis of Peptides with 4-Methylpiperidine as Fmoc Removal Reagent by Solid Phase Synthesis. *J. Mex. Chem. Soc* **2014,** *58* (4), 386-392.

37. Kvach, M. V.; Ustinov, A. V.; Stepanova, I. A.; Malakhov, A. D.; Skorobogatyi, M. V.; Shmanai, V. V.; Korshun, V. A., A Convenient Synthesis of Cyanine Dyes: Reagents for the Labeling of Biomolecules. *Eur. J. Org. Chem.* **2008,** *2008* (12), 2107-2117.