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RESEARCH PAPER

Multiple Triphenylphosphonium Cations as a Platform for the Delivery of a Pro-Apoptotic Peptide

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ABSTRACT

Purpose Triphenyl phosphonium cations (TPPs) are delocalized lipophilic cations that accumulate in the mitochondria of cells. We have explore the effect of increasing the number of TPPs on delivery of a cell-impermeable pro-apoptotic peptide to intact cells.

Methods The pro-apoptotic peptide D-$(KLAKLAK)_2$ (KLA) was extended with 0–3 L-Lysines modified at their ε-amine with TPP. Peptides were studied in HeLa cells to determine their cytotoxic activity and cellular uptake.

Results In HeLa cells, the increased cytotoxicity correlates with the number of TPPs; the peptide with 3 TPP molecules (3-KLA) exerts the highest cytotoxic activity. This FITC-labeled peptide is found to accumulate in intact HeLa cells, whereas peptides with 0–2 TPPs are not detected at the same peptide concentration. Mitochondria-dependent apoptosis of HeLa cells in the presence of 3-KLA was followed by propidium iodide, Annexin-V and DiOC fluorescence by FACS.

Conclusion A facile synthetic methodology has been presented for the delivery of a biologically active peptide into mitochondria of intact cells by attaching multiple TPP moieties to the peptide. This approach was shown to dramatically increase biological activity of the peptide as a pro-apoptotic agent.

KEY WORDS antimicrobial peptide · apoptosis · mitochondria · tripenylphosphonium

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ABBREVIATIONS

Ahx	6-aminohexanoicacid
Alloc	allyloxycarbonyl
FITC	fluorescein isothiocyanate
PI	propidium iodide
TPP	triphenylphosphonium

INTRODUCTION

Mitochondria are unique cellular organelles that are surrounded by two distinct membranes and function as the main powerhouse of the cell. In recent years, several drug delivery systems have been developed for targeting molecules of different size and charge site specifically to either isolated mitochondria (1–3) or to mitochondria of intact cells (4–7). These include liposomes (8–14), metal complexes (15,16), positively charged guanidines (17–20) as well as synthetically designed mitochondria-targeting peptides (21–28) and natural mitochondrial leader peptides (3,29–32). As part of its physiological role in energy production, the mitochondrion generates relatively high levels of reactive oxygen species (ROS), an outcome that has been correlated to a variety of human pathologies. Thus, a variety of antioxidants have been targeted to mitochondria as means of alleviating the oxidative stress localized at these organelles (1,33–38).

Perhaps one of the most studied systems is based on the triphenylphosphonium (TPP) cation molecule. TPP, being a delocalized lipophilic cation (DLC), has the propensity of accumulating in mitochondria, a process primarily driven by the highly negative mitochondrial potential (ψ_{mit}) that succumbs to a negative charge in the matrix (37). This

negative gradient in the mitochondrial membrane potential is even further manifested in tumor cells (39,40).

In a recent report, a TPP molecule conjugated to a nitroxide was successfully utilized to minimize γ-irradiation-induced apoptosis in brain tumor cells (41), highlighting the potential of such TPP-antioxidants in reducing toxicity in post radiation therapy. Furthermore, TPP molecules, in particular those with long alkyl chains, were found to accumulate in mitochondria of rats within a few minutes after i.v. administration (42).

Several studies have also shown that TPP molecules linked to NO-releasing molecules are effective in protecting mitochondria in hypoxic (43) or apoptotic conditions (44). As such, TPP molecules have been shown to be well tolerated in mice (45); they offer a relatively simple approach for targeting a variety of molecules to mitochondria.

Nonetheless, TPP is limited in terms of its cargo type and size. For example, when TPP was conjugated to cell penetrating peptides; these did not localize in mitochondria (46). Furthermore, the use of TPP as a carrier of molecules to mitochondria is not always predictable. Several recent reports have shown that when TPP is conjugated to thiophene fluorophore (47) or to a cyclometaleted Pt(II) complex (48), these conjugates are found mainly in the cytoplasm and nucleoli of cells, respectively.

We have recently reported (49) on the use of multiple TPPs as a means of internalizing a hydrophilic nonapeptide to mitochondria of intact (HeLa) cells. Increasing the number of TPPs (covalently attached to ε-amine of L-Lys) was shown to improve cellular uptake of this model peptide, which was found mostly in the mitochondria.

To further expand the potential of this strategy, we decided to explore the delivery of a well-studied anti-microbial peptide, namely, a 14-mer all-D amino acid peptide of the following sequence: d-KLAKLAKKLAKLAK (KLA). This peptide strongly interacts and disrupts negatively charged membranes as commonly found in bacteria (50). In addition, KLA has been shown to induce mitochondria-dependent apoptosis by its interaction with the mitochondrial membrane. However, KLA's pro-apoptotic activity is limited by its poor uptake into cells. Several strategies have been utilized to increase KLA cellular uptake. This has been accomplished by the addition of tumor-homing peptides (51–55) and cell penetrating peptides (56,57) or by designing KLA mimics with improved cellular uptake and, as a consequence, pro-apoptotic activity in cancer cells (26).

In this paper, we report on the synthesis of KLA peptides with 0–3 Lys-TPP moieties and their cellular uptake and cytotoxicity to cancer cells. We believe that the advantage of this strategy in comparison to others reported thus far relies on its simplicity and versatility (i.e. adding modified L-Lysines onto any given peptide) and to the possibility for optimizing mitochondrial uptake as a function of the number of TPP moieties added.

MATERIALS AND METHODS

Solvents and Reagents

Dry solvents (dichloromethane (DCM), tetrahydrofuran (THF), and N,N-dimethylformamide (DMF), over molecular sieves) were purchased from Acros and used as received. All Fmoc amino acids and coupling reagents, including N-[(1H-benzotriazol-1-yl)(dimethylamino)methylene]N-methylmethanaminium hexafluorophosphate N-oxide (HBTU), N-hydroxybenzotriazole (HOBT) and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), were purchased from GL Biochem (Shanghai). 4-Carboxybutyltriphenylphosphonium bromide, 6-aminohexanoic acid, fluorescein isothiocyanate (FITC), Allyl bromide, $Pd(PPh_3)_4$, and phenylsilane were purchased from Acros. Propidium iodide (PI) and MTT reagent were purchased from Sigma-Aldrich. DiOC was purchased from Molecular Probes (Eugene, OR).

KLA Lys-TPP Synthesis

TPP-modified peptides were synthesized on a solid support using commercially available Rink Amide (MBHA) Resin (0.5 mmol/g, GL Biochem). Peptides were synthesized on a 0.2 mmol scale.

Coupling reactions were performed using 4 equiv. of Fmoc protected amino acid, 4 equiv. of HBTU, 4 equiv. of HOBT and 8 equiv. of DIEA in DMF for 1 h. The coupling reaction was monitored by the Kaiser test. Deprotection of the Fmoc group was achieved using 20% piperidine in DMF (v/v) for 5 min, twice. Efficiency was calculated according to UV absorption at 300 nm of the cleaved Fmoc group ($\varepsilon^{300} = 7{,}800\ cm^{-1}\ M^{-1}$).

The Ahx (6-aminohexanoic acid) linker was protected with Fmoc as previously described (58) and coupled by the same method as described above.

Trityl (trt) group on 1, 2, or 3 ε-amines of L-lysine (for 1–3 L-lysine amino acids, respectively) was deprotected using 5% TFA in DCM for 10 min, and this procedure was repeated 2 additional times. Coupling of the TPP-acid (4-Carboxybutyltriphenylphosphonium bromide) on the free ε-amines (of 1, 2, or 3 L-lysines) was performed in the same way as described for Fmoc-protected amino acids. After the completion of solid phase synthesis, peptides were cleaved and deprotected in a solution of 88:5:5:2 TFA/water/phenol/triethylsilane for 1 h, then precipitated with diethyl ether and spun-down by centrifugation. Peptides were subsequently purified by RP HPLC (Shimadzu LC 2010C, flow rate = 4 mL/min) on a C18 reverse phase column (Phenomenex, Jupiter 300A), using acetonitrile and 0.1% TFA in water as eluents (specific gradients are

detailed in supporting information). KLA-TPP conjugates were monitored at 260 nm. The major peaks were collected, lyophilized and identified by ESI-MS (TSQ Quantum Access MAX, Thermo Scientific) (Figures S1–S11). Purified peptides (KLA with 0–3 Lys-(TPP) at either N or C-termini) were re-injected in an analytical RP-HPLC column (Figures S12-S14). Yields for all peptides were estimated according to crude HPLC chromatograms and are indicated in supporting information.

FITC Labeling

Fmoc-Ahx was introduced on N-terminus of all KLA peptides (KLA with 0–3 Lys-(TPP)) followed by Fmoc deprotection (20% v/v piperidine, 2×5′) and addition of 2 equiv. FITC and 4 equiv. DIEA in 1 ml DMF for 48 h. After peptide cleavage, resulting FITC-labeled KLA peptides were purified by RP HPLC (see Supplementary Material) by the same conditions and monitored at 490 nm (FITC absorption). The major peak was collected and lyophilized.

Synthesis of Fmoc-L-Lys-(TPP)-OH

The C-terminus of Fmoc-L-Lys-(Boc)-OH (1 mmol, 0.47 g) was protected with an allyl group as previously described (59). The Boc group at ε-amine was deprotected using a 2-ml solution of 50% TFA in DCM stirred for 1 h at RT. The solvent was evaporated, followed by coevaporation of residual TFA using DCM and cyclohexane as azeotrope. The resulting oily product was used immediately without further purification.

Synthesis of Fmoc-L-Lys-(TPP)-(OAll)

A mixture of 0.44 g (1 mmol) TPP and 520 mg (2 mmol) PyBOP was dissolved in 2 ml anhydrous DCM and 0.1 ml DMF. Next, 0.7 ml (3 mmol) DIEA was added (pH: 9–10), and the resulting solution was added to the Fmoc-L-Lys-(OAll) (0.4 g, 1 mmol) and stirred for 2 h at RT.

The organic solvent was extracted with brine (5 ml×2), 5% aqueous acetic acid solution (5 ml×2) and water (5 ml×2). The organic layer was collected and dried over $MgSO_4$, and the solvent was evaporated. The resulting oily product was purified by silica gel column chromatography with 0–10% MeOH/$CHCl_3$ as eluents. The compound was identified by ^{1}H NMR (Varian 500 MHz) and ESI-MS. Yield: 80%. ESI-MS: m/z: 753.6 calcd: 753.9

^{1}H NMR ($CDCl_3$): 7.8-7.6 (2xd, 19H TPP aromatic, Fmoc aromatic), 7.4-7.3 (2xm, 4H, Fmoc aromatic), 6.43 (s, 1H, ε-amide of Lys), 5.9 (m, 1H, CH allyl), 5.72 (s, 1H, α-amide of Lys), 5.32-5.21 (dd, 2H, O-CH_2-CH-$\underline{CH_2}$), 4.62-4.61 (d, 2H, CH_2 allyl), 4.3-4.2 (m, 3H, CH_2 Fmoc, CH- α Lys), 4.16 (t, 1H, CH Fmoc), 3.2-3.0 (2xt, 4H, α-CH_2 TPP, δ-CH_2 TPP), 2.27 (t, 2H, ε-CH_2 Lys), 1.86 (m, 2H, δ-CH_2 Lys), 1.64 (m, 2H, β-CH_2 Lys), 1.63 (m, 2H, γ-CH_2 TPP), 1.47-1.46 (m, 2H, β-CH_2 TPP), 1.32 (m, 2H, γ-CH_2 Lys).

^{13}C NMR ($CDCl_3$): 172.69, 172.13, 156.1 (3C, carboxyl), 143.86, 143.75, 141.1 (3C, quat. aromatic), 135.19 (3C, aromatic TPP), 133.2 (6C, aromatic TPP), 131.58 (1C, COO-CH_2-$\underline{C}$H-CH_2), 130.55 (6C, aromatic TPP), 127.62 (2C, aromatic Fmoc), 127.07 (2C, aromatic Fmoc), 125.24 (2C, aromatic Fmoc), 119.8 (2C, aromatic Fmoc), 118.68–117.24 (1C, COO-CH_2-CH-$\underline{C}H_2$), 66.69 (1C, C-α Lys), 65.77 (1C, COO-$\underline{C}H_2$-CH-CH_2), 54.0 (1C, $\underline{C}H_2$-Fmoc), 47.04 (1C, CH-Fmoc), 38.57 (1C, C-α TPP), 34.51 (1C, ε-CH_2 Lys), 31.35 (1C, C-ß Lys), 28.72 (1C, C-ß TPP), 26.07 (1C, δ-CH_2 Lys), 22.32 (1C, γ-CH_2 Lys), 21.87 (1C, δ-CH_2 TPP), 21.45 (1C, γ-CH_2 TPP).

^{31}P NMR ($CDCl_3$): 23.5 (s)

Allyl Deprotection: (Fmoc-L-Lys-(TPP)-OH)

For the reaction, a 25-ml schlenk flask was equipped with magnet stirrer and purged with argon. In a glove box, 800 mg (1 mmol) of Fmoc-L-Lys-(TPP)-(OAll) was dissolved in 4 ml of dry THF. Fifty mg (0.05 mmol) of $Pd(PPh_3)_4$ and 280 µl (2 mmol) phenylsilane were added to the reaction flask. The reaction mixture was stirred at RT under argon atmosphere for 3 h. The reaction mixture was diluted with 20 ml DCM and washed with sat. aqueous NH_4Cl solution (3X20ml). The organic layer was separated, dried over $MgSO_4$, filtered and concentrated in vacuo.

Fmoc-L-Lys-(TPP)-OH was purified by silica gel column chromatography with 0–20% MeOH/$CHCl_3$ as eluents. The pure compound was precipitated from DCM with cold diethyl ether and spun-down by centrifugation, resulting in dry light brown powder. Yield: 95%. The compound was identified by ^{1}H NMR (Varian 300 MHz) and ESI-MS. ESI-MS: m/z: 713.6 calcd: 713.31

^{1}H NMR (CD_3OD): 7.8-7.5 (2xd, 19H TPP aromatic, Fmoc aromatic), 7.3-7.1 (dt, 4H, Fmoc aromatic), 4.3-4.1 (m, 3H, CH2 Fmoc, CH- α Lys), 3.9 (t, 1H, CH Fmoc), 3.2 (m, 2H, α-CH2 TPP), 3.0 (t, 2H, δ-CH2 TPP), 2.1 (t, 2H, ε-CH2 Lys), 1.76-1.2 (m, 10H, δ-CH2 Lys, 2H, β-CH2 Lys, γ-CH2 TPP, β-CH2 TPP, γ-CH2 Lys).

Cell Culture

Cell culture of HeLa cells was maintained in DMEM medium containing 10% fetal calf serum, 1% penicillin-streptomycin, and 1% glutamate and incubated in a humidified atmosphere incubator with 5% CO_2 at 37°C. Cells were routinely subcultured every 2–3 days.

Determination of Cytotoxicity

HeLa cells, maintained in DMEM medium containing 10% fetal calf serum, were plated on a 96-well plate (1.2×10^4 cells/well in 100 μL) and incubated 24 h in a humidified atmosphere incubator with 5% CO_2 at 37°C. The various peptides dissolved in 2% DMSO in PBS were added at different concentration (10–50 μM) and incubated for 3 h in 5% CO_2 at 37°C. Final DMSO concentration was less than 0.2% (v/v).

Next, 20 μl of MTT solution was added to each well and incubated for 2 h in 5% CO_2 at 37°C. The medium was discarded, and 200 μl of DMSO was added. Cytotoxicity was determined by measuring the absorbance at 500 nm on a plate reader (Synergy HT, BIO-TEC). All measurements were performed as triplicates.

Confocal Fluoresence Microscopy

HeLa cells were cultured on μ-slide 8-well plates (Ibidi) 2 days prior to the experiment at 6×10^4 cells/ml. FITC-labeled peptides (2 μM in PBS buffer with 1% EtOH) were incubated with cells for 120 min. After extensive washing with PBS (X3), cells were visualized by confocal microscopy. For colocalization studies, cells were incubated with 15 μM KLA-3-FITC (KLA with 3 Lys-(TPP) and FITC) for 75 min. Mitotracker M7512 (a mitochondrial marker supplied by Invitrogen) at a final concentration of 100 nM was added for another 15 min, and cells were washed three times with PBS. Images were taken with a laser confocal scanning microscope (FluoVIEW FV10i, Olympus). The excitation wavelength for visualization of the FITC- labeled peptide was 448 nm, and emission spectra were collected between 505 and 515 nm. The excitation wavelength for visualization of Mitotracker M7512 was 579 nm, and emission spectra were collected with a long-pass 599 nm filter.

Flow Cytometry with PI and Annexin V

HeLa cells/well (5×10^5) were plated in a 6-well plate (in 5 ml DMED). The plate was incubated overnight at 37°C in 5% CO_2. Twenty μM, 30 μM and 40 μM of 3-KLA were added to cells for 3 h, and the plate was maintained at 37°C in 5% CO_2. Cells were harvested, and 2×10^5 cells were stained with Annexin V and propidium iodide according to the manufacturer's specifications (MEBCYTO apoptosis kit, MBL). Cells were analyzed by FACS (scan-flow cytometer, LSR II).

Flow Cytometry with DiOC, A Mitochondrial Marker

HeLa cells/well (5×10^5) were plated in a 6-well plate (5 ml DMED) and incubated overnight at 37°c in 5% CO_2. 30 μM of 3-KLA was added for 1 h, 2 h and 3 h. The cells were harvested, and 4×10^5 were then incubated with 0.5 nM DiOC for 15 min. Cells were then analyzed by FACS (LSR II).

RESULTS

Synthesis of KLA Peptides with Lys-TPP at Either N- or C-Termini

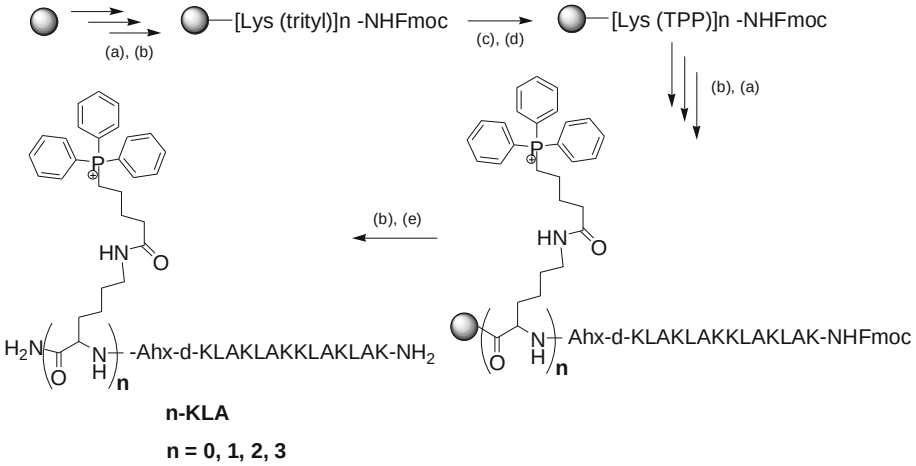
Two synthetic routes were utilized for the conjugation of Lys-TPP monomers at either C- or N-termini of the KLA peptide, respectively. For the introduction of Lys-TPP on the C- terminus, the following synthetic route was used (Scheme 1). Here, the resin was initially loaded with 1, 2 or 3 L-lysines, protected with an acid-labile trityl group on the ε-amine. Next, these trityl groups were removed with a low concentration of TFA (5%), conditions that retain the peptide on the solid support. Subsequently, the free ε-amines (1–3, corresponding to 1–3 L-Lys) were coupled to TPP-COOH by using standard peptide coupling chemistry.

At this stage, after the removal of the Fmoc from the N-terminal amine, a linker was introduced (Fmoc-Ahx), followed by the KLA sequence. As a control peptide, Fmoc-Ahx was introduced onto the resin followed by KLA peptide (denoted as 0-KLA). Final deprotection followed by HPLC purification afforded all four peptides (0, 1, 2, and 3-KLA, where 0–3 to the left of KLA refers to the number of Lys-TPPs and to the attachment of Lys-TPP to the C-terminus of KLA). The Ahx linker was introduced to minimize steric hindrance between the peptide and the TPP moieties.

In a different synthetic approach, we decided to synthesize an L-Lysine monomer that includes the TPP moiety on the ε-amine. This should allow its introduction at either end of peptide by standard Fmoc-based peptide chemistry without the need of trityl deprotection, which might result in some tBOC deprotection, as these two protecting groups are not completely orthogonal.

Scheme 2 presents the synthetic approach for introducing the TPP moiety onto the ε-amine of L-Lysine. The Alloc protecting group on the carboxylic acid was chosen, since it may be cleanly removed with $Pd(PPh_3)_4$ in the presence of Fmoc.

This monomer (Fmoc-L-Lys(TPP)-OH) was then used to prepare the KLA peptide with 0–3 Lys-TPP's at the N-terminus (Scheme 3). This was done in a straightforward manner by standard Fmoc chemistry on the solid support by introducing the KLA peptide followed by the linker (Ahx) and the monomer (added 1–3 times).



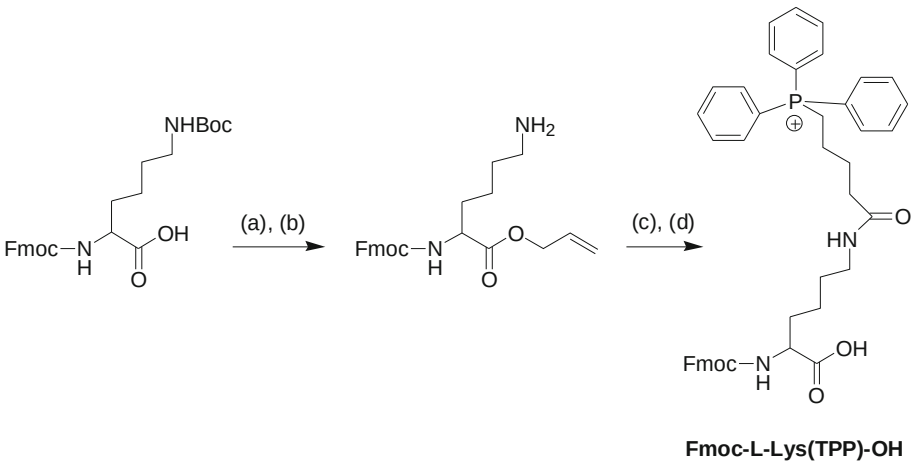
Scheme 1 Solid phase synthesis of n-KLA peptides (where $n = 0$, 1, 2 and 3 Lys(TPP)). Reagents and conditions: (a) Fmoc-amino acid, HATU, HOBT, DIEA; (b) 20% piperidine in DMF; (c) 5% TFA in DCM; (d) 4-Carboxybutyltriphenylphosphonium bromide, HATU, HOBT, DIEA; (e) 88% TFA, 2% triethylsilane, 5% H_2O, 5% phenol, 1 h at RT.

In order to follow cellular uptake of KLA peptides in cells, FITC was introduced at the N-terminus of the KLA series (KLA 0–3) by adding Fmoc-Ahx followed by FITC. The Ahx spacer is critical at this point, since direct labeling of L-Lys by FITC may result in peptide degradation after cleavage from resin (60). Finally, all peptides were cleaved from resin and HPLC purified. Table 1 provides the calculated and expected mass for all KLA peptides. HPLC chromatograms and ESI-MS analysis of purified peptides are provided in Supplementary Material (Figures S1-S14).

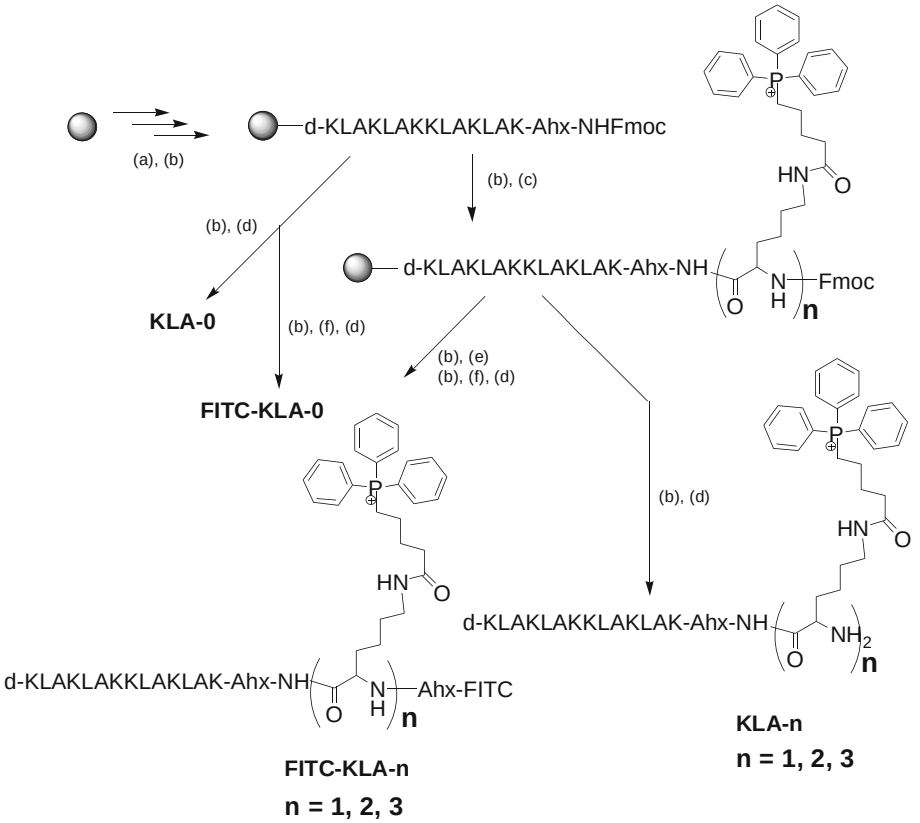
Cellular Uptake of TPP-Modified KLA Peptides

FITC-labeled KLA peptides with 0–3 Lys-TPP moieties (KLA-n-FITC, where n=0-3) were examined for their cellular uptake into intact live HeLa cells. Figure 1a shows the uptake of all KLA peptides at a fixed concentration of 2 μM after adding the FITC-labeled peptides to HeLa cells followed by a short incubation of 120 min. As shown in Fig. 1a, only the KLA peptide with 3 Lys-TPP moieties (KLA-3-FITC) has an appreciable cellular uptake (green fluorescence), whereas all other peptides seem to be excluded from cells.

Next, we were interested in determining whether KLA-3-FITC localizes to the mitochondria. As the FITC signal was relatively weak at 2 μM, we decided to incubate HeLa cells with 15 μM KLA-3-FITC (for 75 min). After this period, Mitotracker was added to cells (for an additional 15 min), and these were imaged by confocal microscopy (Fig. 1b). The image shows a substantial co-localization of KLA-3-FITC and Mitotracker, an observation that is consistent with preferential mitochondrial localization of KLA-3-FITC to mitochondria of intact cells.



Scheme 2 Synthesis of Lys-TPP monomer. Reagents and conditions: (a) Allyl bromide, $NaHCO_3$; (b) 50% TFA in DCM for 1 h at RT; (c) 4-Carboxybutyltriphenylphosphonium bromide, HATU, HOBT, DIEA; (d) $Pd(Ph_3)_4$, phenylsilane.



Scheme 3 Solid phase synthesis of KLA-n peptides (where $n = 0$, 1, 2 and 3 Lys(TPP) with and without FITC. Reagents and conditions: (a) Fmoc-amino acid, HATU, HOBT, DIEA; (b) 20% piperidine in DMF; (c) Fmoc-L-Lys(TPP)-OH, HATU, HOBT, DIEA; (d) 88% TFA, 2% triethylsilane, 5% H_2O, 5% phenol, 1 h at RT; (e) Fmoc-Ahx, HATU, HOBT, DIEA; (f) FITC, DIEA, 48 h at RT.

Cytotoxicity of TPP-Modified KLA Peptides

Cell viability of HeLa cells treated with KLA peptides was determined by the MTT assay. Successful internalization of the KLA peptides into cells is expected to translate into cytotoxicity by an apoptotic mechanism that is mitochondrial dependant (51). According to confocal images shown in Fig. 1, it is expected that the KLA peptides with three TPP moieties would be most active as a result of improved cellular uptake. Indeed, this is the case as shown in Fig. 2.

A 60% mortality of HeLa cells was found after 3 h incubation with 30 μM of 3-KLA at 37°C. At this concentration, all other peptides were by far less toxic. A similar behavior was found for the KLA peptides with 0–3 Lys-TPPs at the N-terminus, namely, peptides KLA-0, KLA-1 and KLA-2 exerted minimal cytotoxicity, whereas

Table I Mass Analysis of KLA Peptides

Peptide	Abbreviation	MW (Calc.)	MW (found)
amide-d(KLAKLAK)$_2$-Ahx-Lys(TPP)	KLA-1	2,108.40	2,108.62
amide -d(KLAKLAK)$_2$-Ahx-2Lys(TPP)	KLA-2	2,581.64	2,581.20
amide -d(KLAKLAK)$_2$-Ahx-3Lys(TPP)	KLA-3	3,054.87	3,054.50
amide -d(KLAKLAK)$_2$ -Ahx-FITC	KLA-0-FITC	2,024.20	2,025.30
amide -d(KLAKLAK)$_2$-Ahx-Lys(TPP)-FITC	KLA-1-FITC	2,610.52	2,611.76
amide -d(KLAKLAK)$_2$-Ahx-2Lys(TPP)-FITC	KLA-2-FITC	3,083.76	3,083.10
amide -d(KLAKLAK)$_2$-Ahx-3Lys(TPP)-FITC	KLA-3-FITC	3,556.99	3,556.04
amide -Ahx-d(KLAKLAK)$_2$	0-KLA	1,635.17	1,635.20
amide -Lys(TPP)-Ahx-d(KLAKLAK)$_2$	1-KLA	2,108.40	2,109.90
amide -2Lys(TPP)-Ahx-d(KLAKLAK)$_2$	2-KLA	2,581.64	2,580.50
amide -3Lys(TPP)-Ahx-d(KLAKLAK)$_2$	3-KLA	3,054.87	3,052.14

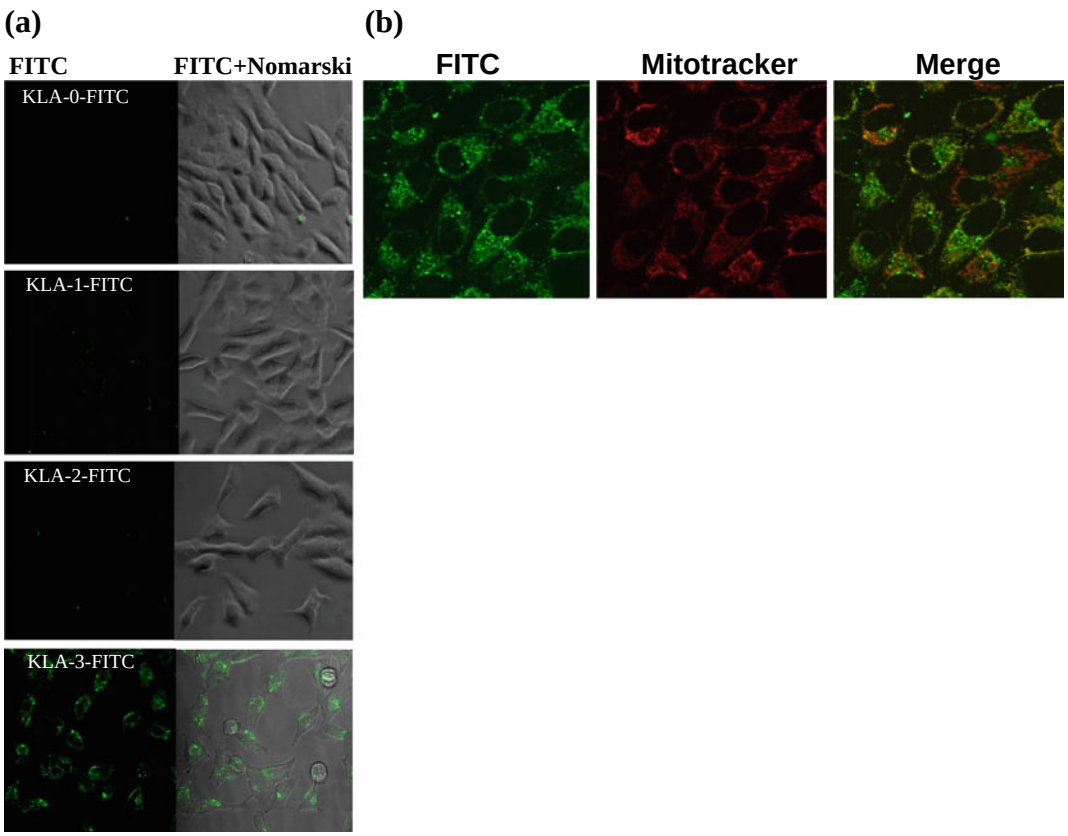


Fig. 1 (**a**) Cellular uptake in HeLa cells of 2 μM FITC-labeled KLA-0-3 peptides. (**b**) Cellular uptake in HeLa cells of 15 μM FITC-labeled KLA-3 (KLA-3-FITC). Mitotracker (100 nM) was added to cells in order to visualize co-localization of KLA-3 with the mitochondrial dye.

KLA-3 resulted in killing 50% HeLa cells at 30 μM (data not shown).

The cytotoxic effect of 3-KLA peptide was further investigated by looking into changes in the cell's membrane integrity, an event that is indicative to early and late apoptotic events. HeLa cells were incubated 3 h at several concentrations of the 3-KLA peptide and subsequently analyzed by FACS.

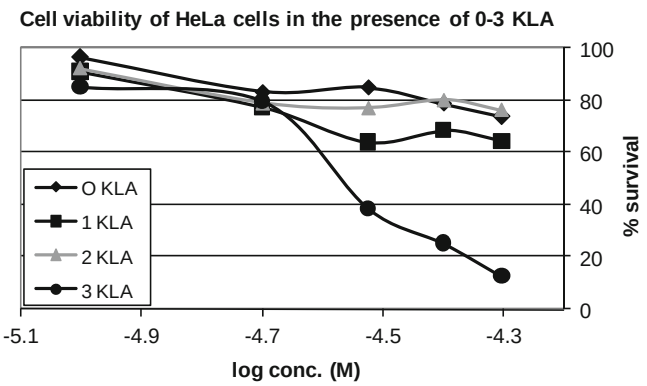


Fig. 2 Cytotoxicity of TPP-modified n-KLA peptides (where $n = 1$, 2 and 3 Lys(TPP)) in HeLa cells as determined by the MTT assay. 0-KLA (no TPP) was used as control peptide**.** Cells were incubated with various peptides for 3 h at RT and then analyzed by the MTT assay for cell viability. Data points represent average of three measurements.

Figure 3 presents the data obtained from both Annexin V and PI fluorescence by FACS. At 30 μM of 3-KLA, most cells are stained by PI (over 70%), and a smaller population is positively stained only by Annexin V (ca. 17%), corresponding to cells at an early apoptotic stage. Interestingly, at 20 μM 3-KLA, most cells are intact with only 18% of the population in early and late apoptotic states.

To further elucidate the mechanism of apoptotic death by 3-KLA, we examined apoptosis in HeLa cells by looking at the fluorescence of a lipophilic dye (DiOC, 3,3′-dihexyloxacarbocyanine) that binds to the mitochondrial membrane and fluoresces according to the given mitochondrial potential (61). Hence, cells that are undergoing mitochondrial-dependent apoptosis lose their mitochondrial membrane potential, an event that may be followed by a shift in fluorescence of DiOC when analyzed by FACS.

This is indeed the case when HeLa cells were treated with 30 μM of KLA-3 and analyzed by FACS (Fig. 4). DiOC florescence was monitored at different time points (after 1–3 h). A gradual increase in the population of cells that show a weaker fluorescence of DiOC was observed, which is consistent with a time-dependent cellular apoptosis

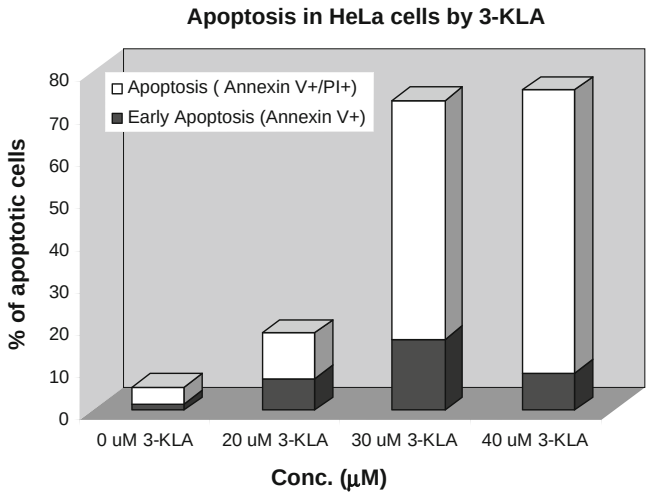


Fig. 3 Following apoptosis induced by 3-KLA in HeLa cells. Cells were incubated with the 3-KLA peptide for 3 h at 37°C followed by FACS analysis with propidium iodide (PI) and Annexin V.

that is mitochondria-dependent. Two major populations were also observed by FACS according to cell size and granulation. These two populations represent apoptotic and viable cells, respectively. (See Supplementary Material for crude FACS data).

Thus, a clear correlation between these two populations and the population of cells that respond to DiOC staining (DiOC-low, Fig. 4) suggests that the main drive into apoptosis results from disruption of the mitochondrial membrane.

DISCUSSION

This report has now expanded the possibility of utilizing multiple TPPs as a means of targeting cell-impermeable peptides (and possibly other molecules) to the mitochondria of intact cells. We have demonstrated a relatively simple synthetic procedure for synthesizing the Fmoc-L-Lys(TPP)-OH monomer (Scheme 2). Using this method, we were able to isolate the desired peptides (e.g. KLA-3, 52% yield) at satisfactory yields (see Supplementary Material). This monomer allows an efficient synthesis of various peptide-TPP conjugates by being compatible with Fmoc-based solid phase synthesis. During the synthesis of the N-terminal TPP-conjugates using Fmoc-Lys(Trt)-OH, we have found that the trityl group is not completely compatible with the tBoc-group and obtained undesired multiple TPP conjugations on the KLA sequence. This obstacle was surmounted by utilizing the Fmoc-L-Lys-(TPP)-OH monomer.

Besides establishing the biological proof of concept of our approach, we have also gained some insight on the capacity of multiple TPP to internalize peptides. In our previous study, we have shown that cellular uptake of a hydrophilic nona-peptide (HA peptide = YPYDVPDYA) into mitochondria of HeLa cells is gradually improved with increasing the number of lipophilic cations (TPP) conjugated to this peptide (49). At a concentration as low as 10 nM, the fully modified HA peptide modified with three Lys(TPP) moieties is already found in HeLa cells as corroborated by confocal microscopy (49). However, the KLA peptide bearing 3 TPP moieties did not show any significant uptake at the nanomolar range (data not shown). Only at a concentration of 2 μM, an appreciable cellular uptake could be detected (Fig. 1a). This difference in cellular internalization between KLA and HA peptides could originate in different hydrophilicities and overall size of these peptides and/or by the inherent propensity of KLA to form a helical fold (26,50). It still remains to be explored the potential of multiple TPPs to internalize larger peptides and to determine the optimal number of TPPs that would result in the most effective cellular uptake of a given cargo.

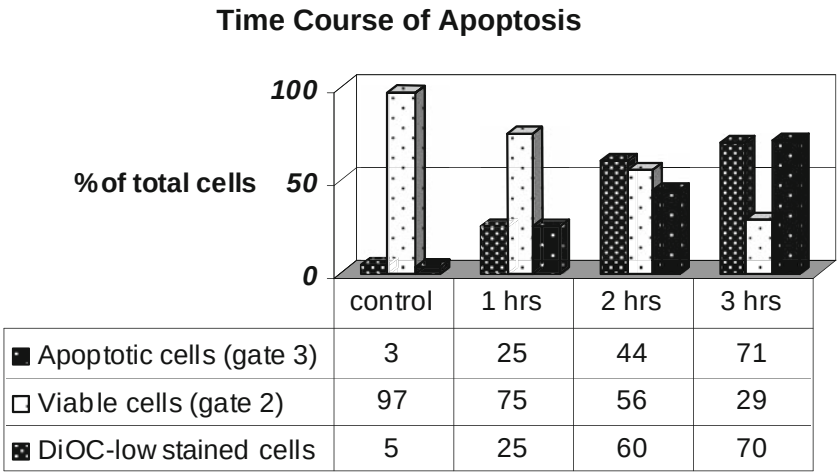


Fig. 4 Following the time-course of apoptosis induced by 30 μM 3-KLA in HeLa cells. Cells were analyzed by FACS after the addition of DiOC. Gate 2 refers to the cell population that was small in size and corresponds to apoptotic cells, whereas gate 3 refers to the viable cells (see Supplementary Material for crude FACS data).

In our present experiments, 3-KLA demonstrated pro-apoptotic activity when incubated with HeLa cells with an IC_{50} value of ca. 25 μM. The cytotoxic effect is observed already after a 1-h incubation of KLA (30 μM, Fig. 4). According to our results, the presence of 3 TPP moieties is crucial for the peptide's cellular uptake and consequently its biological activity. Interestingly, the location of TPP molecules on either N- or C-terminus does not change its cytotoxic ability. Thus, it is likely that adding the Ahx linker to separate the peptide from the Lys-TPP units at either N- or C-termini preserves the biological activity of KLA. We believe that such an approach would also be useful to other biologically active peptides or molecules.

CONCLUSIONS

In this paper, we have explored the potential use of multiple TPP moieties for the internalization of a pro-apoptotic peptide (KLA) into intact cancer cells. We have found significant biological activity only when three TPPs are tethered to either end of this peptide. We have also shown that the well-established activity of this peptide, namely, mitochondrial-dependent apoptosis, is well preserved.

It is now our intention to study the potential therapeutic application of this approach to target other biologically active molecules (preferably to mitochondria) and to determine the capacity of this strategy in terms of the cargo's characteristics such as size and charge.

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