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Synthesis, characterization and anti-cancer activity of a peptide nucleolipid bioconjugate

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ABSTRACT

The synthesis, characterization and anti-cancer activity of a novel peptide nucleolipid bioconjugate is reported in this study. The prerequisite 5′-carboxy derived nucleolipid was synthesized following a five-step solution-phase approach and then coupled to the cytotoxic D-(KLAKLAK)$_2$ sequence by solid-phase bioconjugation. The biophysical and structural properties of the peptide-nucleolipid bioconjugate were evaluated and compared to the peptide controls. These characterization studies revealed that the amphiphilic peptides favored helical-type secondary structures and well-defined nanoparticle formulations that were found to be contributive towards their biological activity. The peptide-nucleolipid bioconjugate displayed greater lethality in comparison to the native D-(KLAKLAK)$_2$AK sequence when treated within the human A549 non-small cell lung carcinoma cell line. Thus, the amphiphilic peptide-nucleolipid forms a new class of anti-cancer peptides that may be developed into promising leads in the fight against cancer.

The so-called "killer" peptide sequence D-(KLAKLAK)$_2$ has been originally designed and developed as an antimicrobial agent.[1] This peptide sequence was found to maintain selective cytotoxicity towards gram positive and negative bacteria, but not in mammalian cell types. The chemical basis for its selectivity has been attributed to its poly(cationic) amphiphilic nature, which facilitates cell translocation across the negatively charged bacterial membrane, but with limited permeability across the zwitterionic lipid bilayer of mammalian cell types.[2] Once internalized within cells, the positively charged D-(KLAKLAK)$_2$ sequence has been found to accumulate on the surface of the mitochondria causing dissipation of the negatively charged mitochondrial membrane potential.[3] This depolarization resulted in a loss of mitochondrial integrity which caused membrane rupture and the release of mitochondrial contents into the cytoplasm. The release of the cell death effectors from the mitochondria, including cytochrome c, the second mitochondrial-derived activator of caspase (Smac/DIABLO) and the apoptosis-inducing factor (AIF) ultimately resulted in programmed cell death.[4] In an effort to exploit the cell death effects of D-(KLAKLAK)$_2$, modifications to the primary sequence[5] and conjugation with cell penetrating/targeting peptides and proteins[6–14] have resulted in improved cell translocation and cytotoxic activity within malignant mammalian cell types, such as in human cancers. In spite of these successful examples, effective methods for the safe and long-lasting administration of peptide based therapeutics are still in widespread demand. Limitations such as poor peptide cell permeability, minimum exposure and resident time at the target site for activity have halted the translation of bio-active peptides such as the D-(KLAKLAK)$_2$ sequence from pre-clinical to clinical use. In an effort to mitigate these limitations and improve the 'drug-like' properties of the pro-apoptotic D-(KLAKLAK)$_2$ sequence, a chemically robust and structurally pre-organized amphiphilic nucleolipid is proposed to enhance cell permeability and mitochondria localization in tumors, resulting in potent and long-lasting anti-cancer effects.

The nucleolipids represent an interesting class of bioconjugates owning the ability to form stable, higher-ordered structures for potential applications in medicinal chemistry.[15,16] For example, they have been shown to form complexes with short-interfering RNA (siRNA), facilitating siRNA transfection leading to the down-regulation of mRNA and protein expression in cancer cells.[17–19] Moreover, we have recently reported the synthesis, GRP78 oncogene binding and selective anti-cancer activity of a novel aminoacyl nucleolipid within the SR human leukemia cancer cell line.[20] Thus, the nucleolipids have effectively served as robust delivery vehicles for biologicals and as potent cytotoxic agents for enhancing the cell death response in cancer. In this study, a thymidine-derived nucleolipid is rationally designed to contain a reactive carboxy group for coupling with the pro-apoptotic

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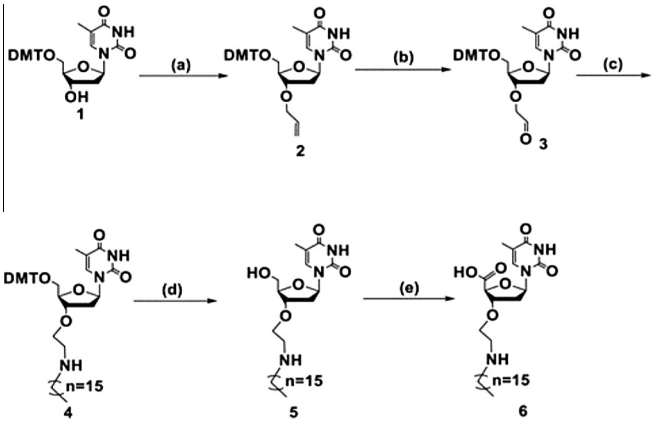
D-(KLAKLAK)$_2$ sequence, thereby affording the desired peptide-nucleolipid bioconjugate (Fig. 1A). The thymidine nucleoside provides a chemically resilient and structurally pre-organized scaffold,[21] while the long chain alkylamine is anticipated to enhance cell permeability and mitochondria localization of the D-(KLAKLAK)$_2$ sequence for potent anti-cancer activity.

The synthesis of nucleolipid **6** (Scheme 1) was initiated by the regioselective attachment of the allyl group at the 3′-hydroxyl of commercially available 5′-*O*-(4-dimethoxytrityl) DMT-thymidine, **1**. The allylation reaction proceeded smoothly with sodium hydride (NaH) as base, and allyl bromide as alkylating reagent resulting in 87% yield of the mono-allylated nucleoside, **2**, and without any observable allylation on thymine. The allyl group in **2** was then oxidized to the aldehyde, **3**, and isolated in 84% yields following silica gel column chromatography. The reactive 3′-aldehyde was subsequently condensed with hexadecylamine and reduced using sodium cyanoborohydride to yield the alkylamine –derived thymidine, **4**. This two-step one-pot reductive amination reaction favored the formation of the desired intermediate, **4**, in 43% yields. In this reaction, the conversion of the imine to secondary amine was closely monitored by ^{1}H NMR which confirmed the disappearance of the iminium proton (δ: 6.85 ppm) upon complete reduction of the Schiff base. Following quantitative removal of the 5′-DMT group using trichloroacetic acid (TCA), a TEMPO-mediated oxidation reaction converted the 5′-hydroxy to the carboxylic acid, **6**, in 50% yield. Each reaction intermediate and the final product, **6**, were characterized by NMR, IR spectroscopy and by MS to confirm purities and identities (see Supporting Information).



Scheme 1. Synthesis of 5′-carboxy-derived thymidine nucleolipid, **6**. Conditions: (a) allyl bromide, NaH, THF, sonication, rt, 3 h, 87%, (b) 4% OsO_4 in *t*-BuOH, NMO, $Na_2S_2O_5$, $NaIO_4$, acetone:phosphate buffer (3:1 v/v), 84%, (c) (i) 1-hexadecylamine, THF, rt, 20 min, (ii) $NaBH_3CN$, THF, rt, 4 h, 43% (d) 3% TCA:DCM, rt, 4 h, >99%, (e) (i) TEMPO, BAIB, DCM, rt, 2 h, (ii) MeCN:H_2O, rt, 24 h, 50%.

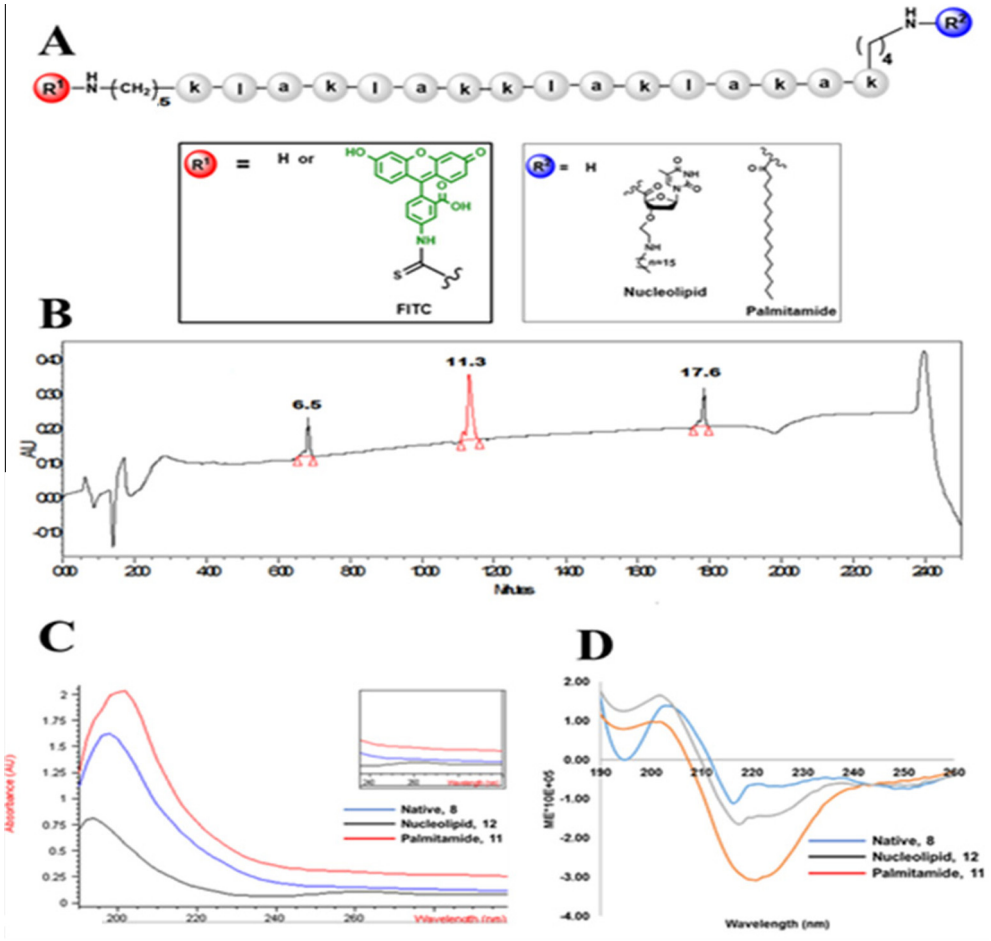


Figure 1. Biophysical and structure properties of the D-(KLAKLAK)$_2$-AK analogues. (A) Peptide structures. The D-(KLAKLAK)$_2$-AK peptide scaffold with R^1(red) = H or FITC, R^2(blue) = H, Nucleolipid or Palmitamide. R^1, R^2 = H, **8**, R^1 = FITC, R^2 = H, **9**, R^1 = H, R^2 = Palmitamide, **11**, R^1 = H, R^2 = Nucleolipid, **12**, R^1 = FITC, R^2 = Palmitamide, **13**, R^1 = FITC, R^2 = Nucleolipid, **14**. All amino acids are in their D-configuration. (B) Comparison of the retention times of D-(KLAKLAK)$_2$-AK peptides on a C_{18} column by RP-HPLC. (C) UV/Vis spectroscopy (190–300 nm) of the peptides. Inset shows the nucleolipid-derived D-(KLAKLAK)$_2$-AK, **12**, absorbance at 260 nm. (D) CD spectra of peptides at 12.5 μM in H_2O: TFE (50:50, v/v).

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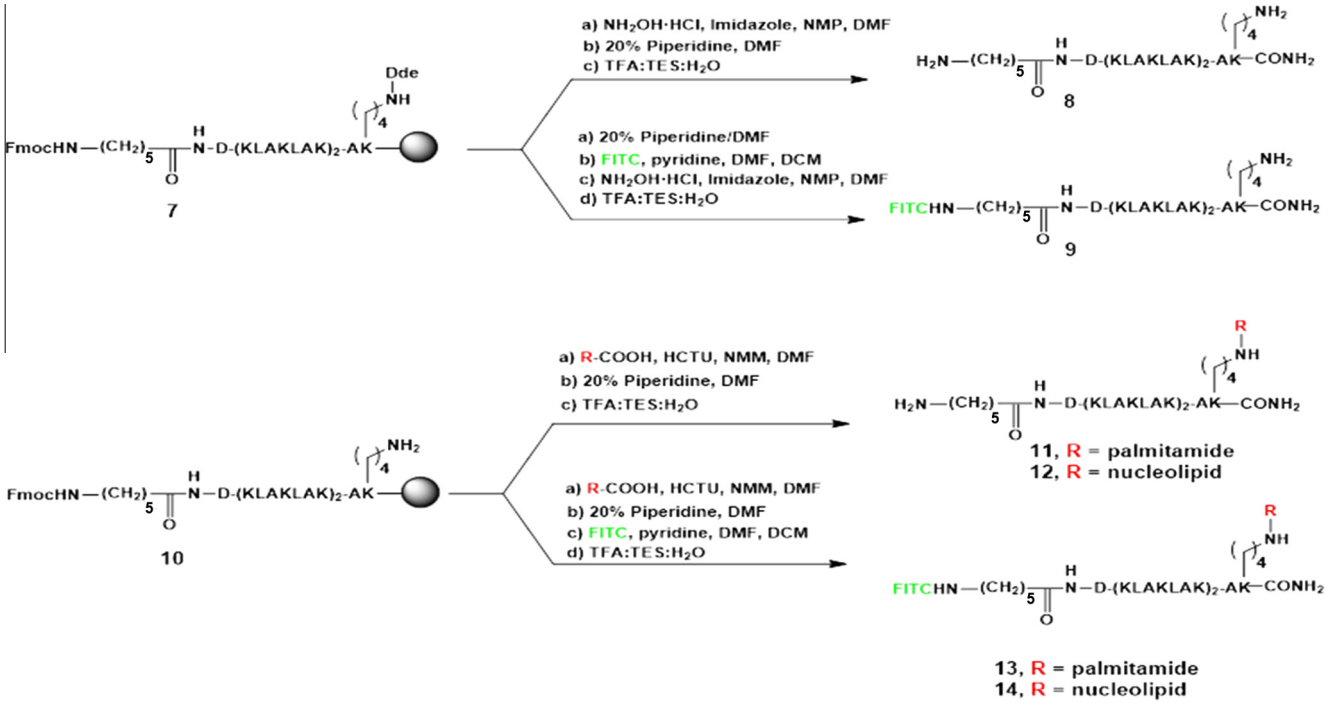
The synthesis of the D-(KLAKLAK)$_2$-AK sequence, **8**, was accomplished by conventional Fmoc-solid phase peptide synthesis on a Rink amide hydrophilic poly(ethylene glycol) resin (Nova PEG, 0.47 mmol/g).[22] At the *N*-terminus of the peptide sequence an aminohexanoic acid (AHx) linker was attached for the incorporation of fluorescein isothiocyanante (FITC).[23] Following cleavage and deprotection, the FITC-labeled peptide sequence, **9**, was isolated by RP-HPLC for characterization studies. At the *C*-terminus, an orthogonally protected Lys(Dde) residue was selectively deprotected on solid-phase using hydroxylamine hydrochloride buffered with imidazole. These mild conditions have been shown to cleave the Dde group without concomitant Fmoc-deprotection which has been shown to occur when hydrazine is used as Dde deblocking reagent.[24] Bioconjugation was first attempted by coupling palmitic acid at the *C*-terminus of the partially deprotected peptide sequence, **10**, bound to the solid support (Scheme 2). Optimized conditions for peptide coupling reactions using HCTU as coupling reagent and NMM as base were adopted for making the desired bioconjugates on solid support. The palmitamide-derived D-(KLAKLAK)$_2$-AK sequence was Fmoc-deprotected and either coupled with FITC at the *N*-terminus, followed by cleavage and deprotection from solid-phase, or was directly cleaved and deprotected for LCMS analysis and purification. The palmitamide-derived D-(KLAKLAK)$_2$-AK sequence, **11**, and the FITC-labeled sequence, **13**, were isolated by RP-HPLC and their identities were confirmed by MS. These coupling conditions were adopted for the synthesis of the nucleolipid-derived D-(KLAKLAK)$_2$-AK sequence, which also produced, respectively **12** and **14**, without and with the FITC label. In either cases, the peptide bioconjugates were isolated in sufficient yields (10–33%) and purities ($\geqslant$96%) (Table S1, see Supporting Information) for exploring biophysical, structure and biological properties.

The peptide bioconjugates were anticipated to display greater hydrophobicity relative to the native D-(KLAKLAK)$_2$-AK sequence. The enhanced hydrophobic character of cell-penetrating peptides has been shown to improve their membrane translocation, presumably due to the favorable non-polar interactions in between the hydrophobic side-chain residues of the peptides and the long-chain alkyl groups found within the lipid bilayer.[25] Comparison of the retention times on RP-HPLC for the native, D-(KLAKLAK)$_2$-AK sequence, **8**, RT = 6.5 min, with the palmitamide-derived D-(KLAKLAK)$_2$-AK, **11**, RT = 17.6 min, and the nucleolipid-derived D-(KLAKLAK)$_2$-AK, **12**, RT = 11.3 min, confirmed that the palmitamide, followed by the nucleolipid produced the greatest hydrophobic effects onto the native D-(KLAKLAK)$_2$-AK sequence (Fig. 1B).

The hydrophobicity of the peptide bioconjugates was also confirmed by the DLS zeta potential measurements and the logP values evaluating the octanol:water partition coefficients of the FITC-labeled peptides at 490 nm (Table S2, see Supporting Information). Furthermore, UV–Vis spectroscopy also confirmed the characteristic absorption bands of the peptide bonds ($\lambda_{max} \sim 220$ nm) and the thymine base ($\lambda_{max} \sim 260$ nm) corresponding to the nucleolipid-derived D-(KLAKLAK)$_2$-AK, **12**, (Fig. 1C). CD spectroscopy was then used to evaluate the secondary structures (if any) of the peptides in H_2O:TFE. The combination of H_2O:TFE has been especially useful in mimicking the amphiphilic lipid bilayer microenvironment which has served to stabilize peptide secondary structures in solution.[26] In this study, the native, D-(KLAKLAK)$_2$-AK sequence, **8**, displayed a stable, α-helical secondary structure (65% helicity), while the palmitamide-derived D-(KLAKLAK)$_2$-AK, **11**, displayed enhanced helicity (76%) and the nucleolipid- derived D-(KLAKLAK)$_2$-AK **12**, demonstrated a less stable (42%) α-helical secondary structure (Fig. 1D and Table S2). Many amphiphilic cell penetrating peptides adopt α-helical secondary structures. In certain cases, this peptide motif functions as a recognition site with other membrane spanning proteins (such as the pore proteins) and facilitates cellular translocation activity.[27] The amphiphilic α-helical peptides, **8**, **11** and **12**, were subsequently analyzed by DLS and TEM to determine ionic charge, size, shape and particle distributions (Fig. 2). The DLS data (Table S2, see Supporting Information) confirmed positively charged peptide ionic structures according to the zeta-potential measurements obtained in H_2O. The size distributions



Scheme 2. Synthesis of FITC and non-FITC tagged D-(KLAKLAK)$_2$-AK conjugated with nucleolipid **6** or palmitamide.

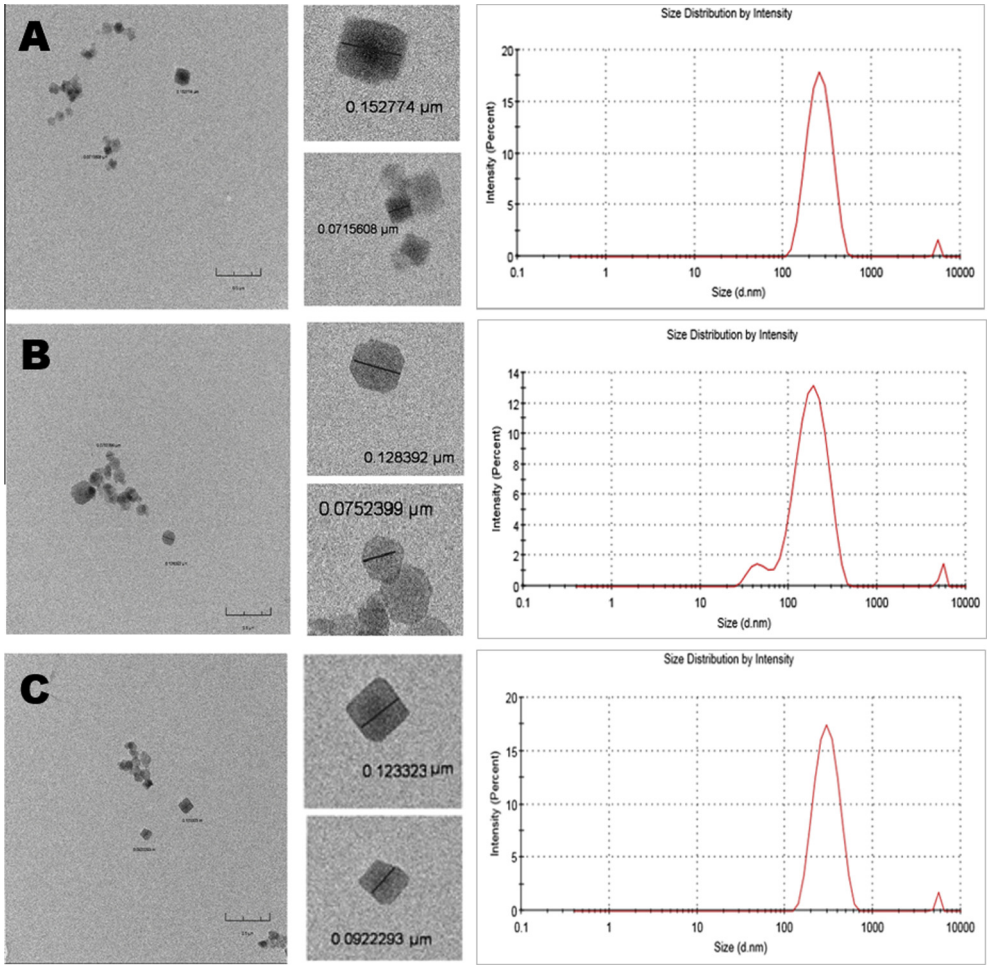


Figure 2. TEM images and DLS particle diameter size distributions. (A) The native D-(KLAKLAK)$_2$-AK sequence, **8**, (B) palmitamide-derived D-(KLAKLAK)$_2$-AK sequence, **11**, and (C) the nucleolipid-derived D-(KLAKLAK)$_2$-AK sequence, **12.**

(60–300 nm) detected by DLS and TEM were found to be comparable and indicated a single, major population of nanoparticles for each formulation (Fig. 2).

For example, the native D-(KLAKLAK)$_2$-AK sequence, **8**, displayed, uniform, square shaped particles ranging in sizes from 70 to 270 nm. The palmitamide-peptide bioconjugate, **11**, produced smaller, spherical-shaped particle sizes (70–190 nm), while the nucleolipid-peptide bioconjugate **12**, demonstrated the largest particle size distribution (60 – 310 nm). Considering the self-assembly and nanoparticle formulation of similar amphiphilic D-(KLAKLAK)$_2$ sequences have led to significant anti-cancer effects,[28] the cancer cell line toxicities of the peptides were subsequently evaluated.

A lethality assay was performed against a panel of 60 cancer cell lines at National Cancer Institute (NCI).[29] A single dose screen was performed with the native, D-(KLAKLAK)$_2$-AK sequence, **8**, (10 µM), the palmitamide-derived D-(KLAKLAK)$_2$-AK sequence, **11**, (7.25 µM), and the nucleolipid-derived D-(KLAKLAK)$_2$-AK sequence, **12**, (2.13 µM). The cell growth data (see Supporting Information) indicated that the peptides were found to be most active towards a panel of non-small cell lung carcinoma (NSCLC). Interestingly, the palmitamide and nucleolipid-derived D-(KLAKLAK)$_2$-AK sequences, **11** and **12**, showed the highest toxicity (~30%) within the A549 NSCLC cell line, resulting in a 1.5-fold enhancement relative to the native D-(KLAKLAK)$_2$-AK sequence, **8**, (~20%) (Table 1). These results correlate nicely with the recently reported anti-cancer effects of the amphiphilic D-(KLAKLAK)$_2$-phospholipid liposome formulation which enhanced the cytotoxicity of the killer peptide sequence within the A549 lung cancer cells and within the drug-resistant lung cancer A549/Taxol cell line.[30] Thus, the amphiphilic palmitamide or nucleolipid-derived D-(KLAKLAK)$_2$-AK sequences represents an interesting class of bioconjugates that have served to potentiate the cancer cell death response of the native D-(KLAKLAK)$_2$-AK within the A549 lung cancer cell line. Furthermore, the cytotoxic selectivity for cancer cell lines over normal tissues for bioconjugates **11** and **12** is unanticipated but may be introduced by the incorporation of cancer cell targeting ligands.[6,7,9,12–14] This is currently a focal point in our research program geared towards the development of cancer-targeting peptide bioconjugates.

Table 1
NCI-60 cancer cell line screen lethality (% cell death) data for a selection of human Non-Small Cell Lung Cancer cell lines

Lethality (% cell death) in NSCLC			
Sequence	A549	NCI-H226	NCI-H522
Native, **8**	19	1	14
Palmitamide, **11**	32	0	16
Nucleolipid, **12**	31	4	11

In conclusion, the synthesis, characterization and anti-cancer activity of a new class of killer peptide bioconjugates are reported in this study. The peptide bioconjugates were found to be more hydrophobic relative to the native sequence. Moreover, the peptide

bioconjugates displayed α-helical structures and cationic nanoparticle formulations that were contributive towards their anti-cancer activities. Within a NCI-60 cancer cell line screen, the peptide bioconjugates displayed enhanced cell death activity relative to the native sequence within the human A549 lung cancer cell line. These results validates the anti-cancer utility of the new amphiphilic peptide bioconjugates and paves the way for a cancer cell biology study that may ultimately unveil their mechanism of action.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.06.020.

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