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Synthesis, DNA binding and anti-leukemic activity of an aminoacyl nucleolipid

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ABSTRACT

The synthesis and characterization of a new class of DNA binding molecule exhibiting potent and selective anti-leukemic activity is described. The synthesis of an aminoacyl nucleolipid was developed from an efficient EEDQ coupling strategy, in which a series of seven bioconjugates were synthesized in yields of 53–78%. Guanosine bioconjugate **7**, was used as building block for the synthesis of a target aminoacyl nucleolipid **14**. Its GRP78 DNA binding affinity was confirmed by gel shift assay, CD spectroscopy, T_m measurements and dynamic light scattering experiments. Moreover, in a single dose (10 μM) screen against a panel of 60 cancer cell lines, aminoacyl nucleolipid **14** was found to selectively trigger greater than 90% cell death in a SR human leukemia cancer cell line. The reported aminoacyl nucleolipid represents a useful model for a new class of DNA binding molecules for the development of potent and selective anti-cancer agents.

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Aminoacyl nucleolipids represent an interesting class of bioconjugates owning the ability to form stable, higher-ordered structures for potential applications in medicinal chemistry.[1a] For example, they form complexes with short-interfering RNA (siRNA), applicable in siRNA transfection leading to the down-regulation of mRNA and protein expression in cancer cells.[1b–d]

These bioconjugates may also have the ability to bind and stabilize double-stranded DNA by a series of non-covalent interactions. These include, H-bond base-pairing and π-stacking of the nucleoside base, as seen in the canonical Watson–Crick[2a] and Hoogsteen base-pairing interactions of ligands with duplex DNA.[2b,c] Favorable ionic interactions can occur between the positively charged aminoacyl and the anionic phosphodiester DNA backbone as illustrated by the condensation of DNA with poly-Arg and poly-Lys sequences.[3] Lastly, the van der Waals/hydrophobic interactions of the lipids create an amphiphilic microenvironment for DNA complex formation and cell permeability[4] (Fig. 1). In the fight against cancer, stable DNA-bioconjugate interactions may form the basis for an effective gene therapy strategy.[5]

The glucose-regulated protein 78 (GRP78) is a chaperone protein that functions to regulate protein folding events in the lumen of the endoplasmic reticulum (ER) of all cell types.[6] In cancer, GRP78 is overexpressed and exclusively localized on the cell surface where it exhibits a variety of signal transduction pathways associated with tumorogenesis and resistance towards apoptosis.[7]

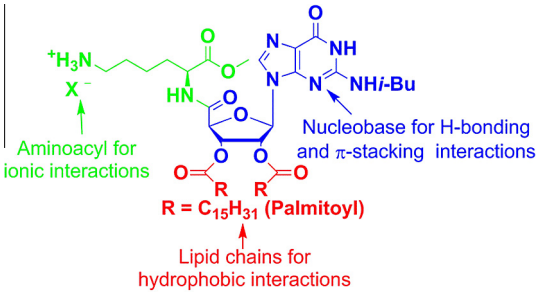

Figure 1. Chemical structure and DNA-binding interactions of aminoacyl nucleolipid, **14**.

Thus, GRP78 functions as an ideal molecular marker for the selective detection and treatment of cancer cells.[8]

In an effort to develop potent GRP78 DNA binders for anticancer applications, we describe the development of an aminoacyl nucleolipid, **14**. This aminoacyl nucleolipid, as a valuable representative of a new class of DNA binding molecules has been validated by its ability to bind to the promoter region of GRP78 DNA, while soliciting potent and selective anti-leukemic activity.

The synthetic strategy begins with the generation of *N*-isobutyrroyl 5'-carboxy 2′,3′-bis-*O*-(carbobenzyloxy) guanosine, **4**, (Scheme 1) as the building block component for furnishing bioconjugates (Table 1). In the preparation of **4**, *N*-isobutyrroyl 5′-dimethoxytrityl guanosine, **1**, was used as commercially available starting material to initiate the synthesis strategy. The Cbz

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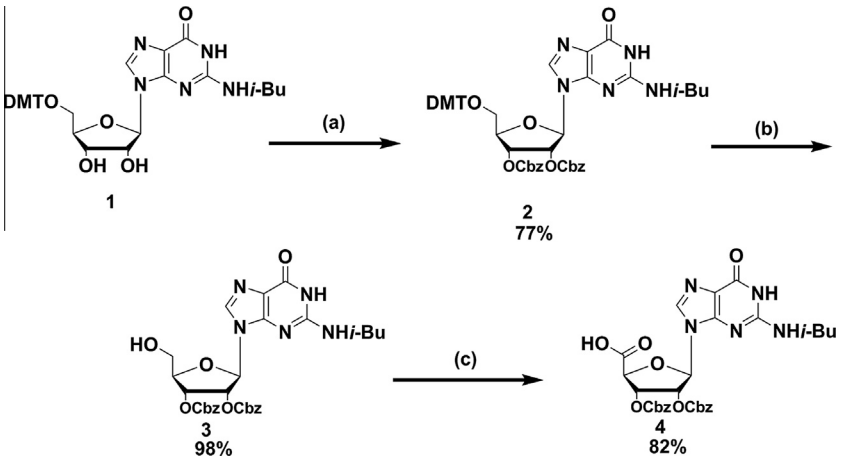
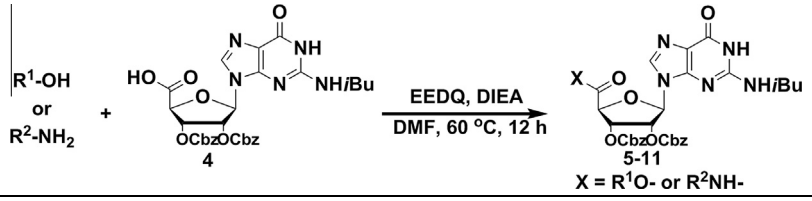
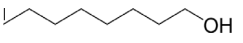
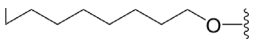
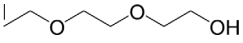
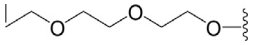
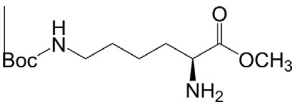
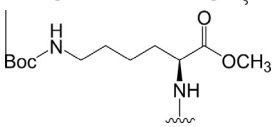
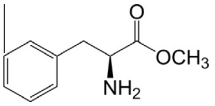
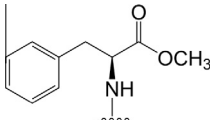
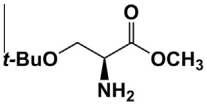
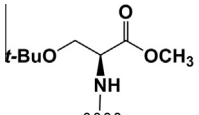
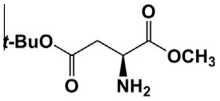
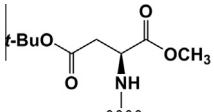
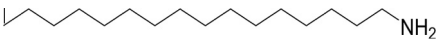
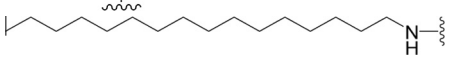


Scheme 1. Synthesis of *N*-isobutyrroyl 5′-carboxy 2′,3′-bis-*O*-(carbobenzyloxy) guanosine, **4**. Reagents and conditions: (a) DMAP, benzyl chloroformate, DCM, 0 °C then rt, 72 h, 77%; (b) 3% TCA:DCM, rt, 15 min, 98%; (c) TEMPO, BAIB, MeCN:H_2O (50:50 v/v), rt, 15 h, 82%.

Table 1
Bio-conjugation of aminoacyl and lipid nucleosides

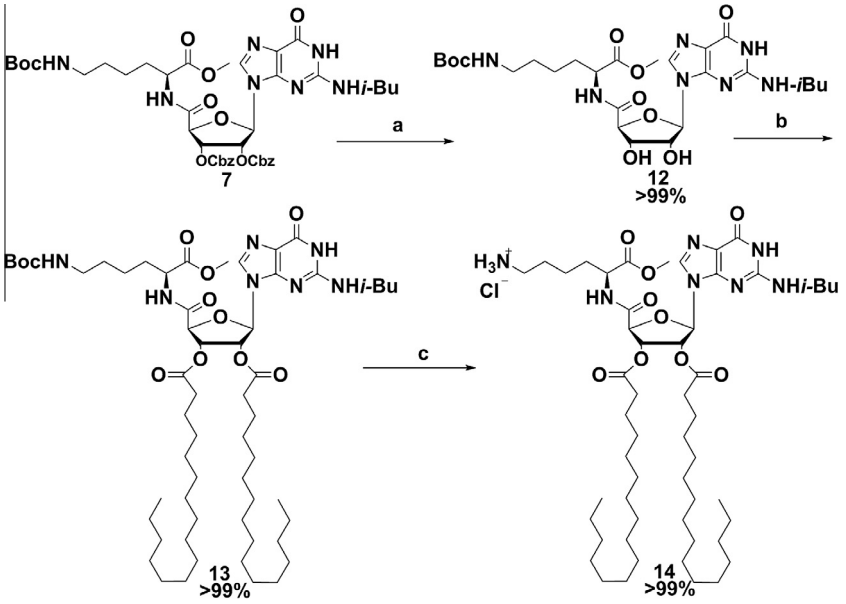


Entry	R^1-OH or R^2-NH_2	X	Yield (%)
5			73
6			55
7			53
8			78
9			67
10			70
11			70

protecting groups were installed using an excess of benzylchloroformate and DMAP in DCM.[9] Following carbomylation, **2**, was detritylated in 98% yield using 3% trichloroacetic acid in DCM.[10] *N*-isobutyrryol-2′,3′-bis-*O*-(carbobenzyloxy)guanosine, **3**, was oxidized in 82% yield using a TEMPO oxidation to the corresponding carboxylic acid, **4**.[11]

With building block **4** in hand, a small library of aminoacyl and lipid conjugates were prepared. Coupling conditions were optimized based on literature precedence,[12] by using *N*-ethoxycarbonyl 1,2-ethoxy-1,2-dihydroquinoline, EEDQ, (2 equiv) as coupling reagent and *N,N*-diisopropylethylamine, DIEA (2 equiv) as the base in DMF. Briefly, alkyl amines or alcohols (Table 1, entries 5, 6, 11) were coupled with **4** to their corresponding amides or esters, respectively, in yields of 55–73%. In similar fashion, amino esters (Table 1, entries **7**–10) were coupled to their corresponding amides with building block **4** in yields ranging from 53% to 78%. The pure bio-conjugates were characterized by NMR, IR, MS and polarimetry (see Supplementary data).

Bioconjugate **7**, was selected for the preparation of aminoacyl nucleolipid, **14**, following a simple three-step procedure



Scheme 2. Synthesis of aminoacyl nucleolipid, **14**. Reagents and conditions: (a) H_2, 10% Pd/C, EtOH, rt, 1.5 h, >99%; (b) palmitoyl chloride, DMAP, py, 2 h, >99%; (c) 50% TFA:DCM, 2 h, rt, then 1 M HCl, 3 h, >99%.

(Scheme 2). Firstly, the Cbz protecting groups were removed following traditional hydrogenation conditions,[9] generating diol **12** in quantitative yields. The product was dissolved in pyridine and acylated with an excess of palmitoyl chloride[13] to afford the protected aminoacyl nucleolipid **13** in >99% yield. Following purification, the Boc-protecting group of **13** was removed using 50% TFA:DCM, followed by salt exchange with aqueous HCl. The desired product, **14**, obtained in quantitative yield was lyophilized to dryness and characterized (see Supplementary data). Attempts to selectively deprotect the guanine NH-*i*-Bu using mildly basic conditions[14] resulted in concomitant ester cleavage of the palmitoyls and methyl ester groups. Thus, we rationalized that partial protection of aminoacyl nucleolipid, **14** may confer greater resistance towards degradation, while inhibiting formation of higher-order self-assemblies (i.e. guanine-tetraplexes) that may influence DNA binding and biological activity.[15] CD spectroscopy of aminoacyl nucleolipid **14** (0.2–3.9 mM) did not exhibit a guanine-tetraplex structure (see Supplementary data, Fig. S2). They are usually characterized by an intense positive band near 264 nm and a negative one near 245 nm (for parallel tetraplex structures), and a positive band near 290 nm and negative ones near 260 and 245 nm (for anti-parallel tetraplex structures).[16]

The promoter region of GRP78 DNA encompassing nucleotides 252–269[17] was synthesized by conventional automated solid-phase oligonucleotide synthesis.[18] The oligonucleotides were purified by reverse-phase ion-pairing HPLC (RP-IP HPLC)[19] and characterized by mass spectrometry (see Supplementary data, Fig. S1). Complementary sequences (0.57 μM) were annealed in 15 μL of 30% sucrose in 5× TAE buffer (89 mM Tris/acetate, 2.5 mM EDTA, pH 4.2) and incubated at 37 °C for 36 h with and without ligand **14**, at 0, 5, 25, 50, and 100 equiv, based on the ratio of **14**/DNA phosphate groups, which corresponds to the following concentrations: 0, 0.2, 0.9, 1.9, 3.9 mM, respectively. A 24% native PAGE gel shift binding assay was performed to evaluate ligand binding to GRP78 DNA (Fig. 2). Following electrophoresis, gradual disappearance of the DNA bands was visualized by UV-shadowing at 260 nm. The observed trend is typical of higher-order complex structures, which results in the diminished intensity of the UV-bands corresponding to duplex DNA.[20] Quantitative analysis of the ratio

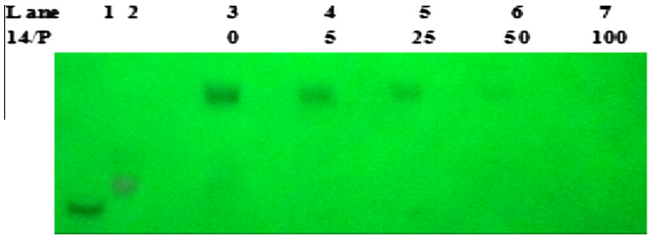


Figure 2. Gel shift assay for GRP78 DNA with 14: from lane 3 to 7, increasing amount of ligand **14** from 0 to 100 equiv. Compound **14**/P (0–3.9 mM). Lane 1 and 2 are single stranded GRP78 DNA.

between duplex DNA with and without **14** demonstrated 50% duplex binding affinity (K_D) at 0.7 mM.

Next, the GRP78 DNA binding stability with and without **14** was evaluated by thermal denaturation (Fig. 3). Changes in duplex DNA UV-absorption at 260 nm was monitored with increasing temperatures (5-90 °C). DNA samples (1.1 μM) were prepared by annealing complementary single strands in physiological phosphate binding buffer (140 mM KCl, 1 mM $MgCl_2$, 5 mM Na_2PHO_4 adjusted to pH 7.2). The melting temperature (T_m) of native GRP78 DNA was evaluated at 66 °C, whereas the addition of ligand (0.2–3.9 mM) produced drastic changes in the DNA melting curves. For example, at 0.2 mM, **14** exhibited a stabilizing effect on GRP78 DNA duplex (ΔT_m = +4 °C), whereas at higher concentrations (0.9–3.9 mM) changes in duplex DNA hyperchromicities with increasing temperatures were indicative of higher-order structure transitions.[21]

In order to gain greater insight into the structural changes of native GRP78 DNA with the addition of ligand **14**, the circular dichroism (CD) spectrum was collected (Fig. 4). The samples were prepared as previously described for the thermal denaturation studies. The native GRP78 DNA duplex exhibited a CD structure consistent with a B-type helix, with characteristic broad maximum band between 260 and 280 nm and a minimum at 248 nm.[22] The amplitudes of the molar ellipticities at these characteristic wavelengths were found to diminish upon titration with **14** (0.2–3.9 mM) suggesting that the ligand-bound structure compromises DNA duplex helicity until the formation of a higher-order complex

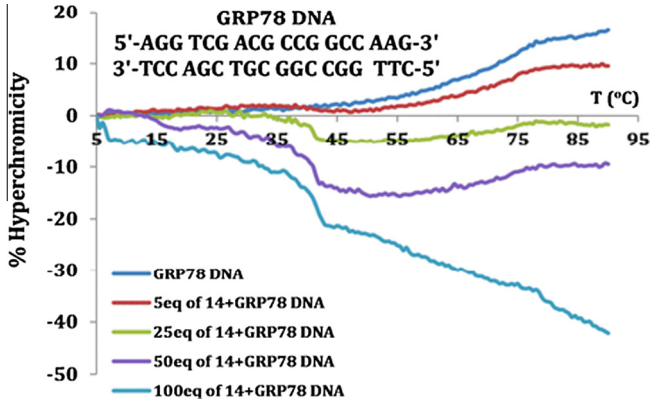


Figure 3. Thermal denaturation curves. Changes in % hyperchromicity at 260 nm versus temperature of GRP78 DNA (1.1 μM) in the presence of ligand **14** (0.2–3.9 mM).

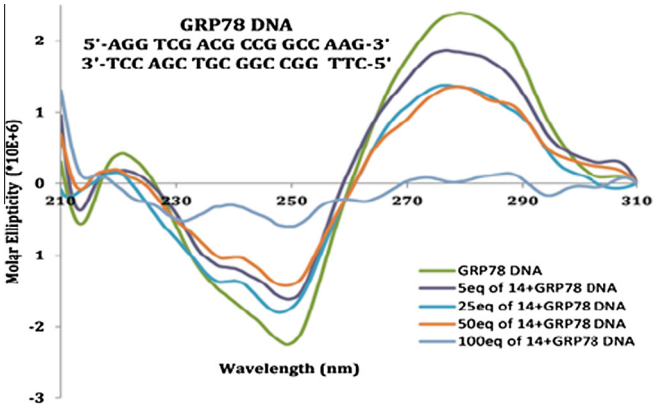


Figure 4. Circular dichroism (CD) spectrum of GRP78 DNA (1.1 μM) with complexing ligand **14** (0–3.9 mM) in phosphate buffer.

persists at higher ligand concentrations (i.e. 100 equiv **14**/P, 3.9 mM).

To further characterize the size of the complex formed between GRP78 DNA and **14**, dynamic light scattering (DLS) studies were performed relative to native DNA and aminoacyl nucleolipid **14**. DNA (1.1 μM) was prepared with and without aminoacyl nucleolipid **14** (3.9 mM) and incubated in phosphate buffer for 36 h prior to DLS measurements (Table 2). The observed effective diameters for the particle sizes shows fixed values for DNA (~1.8 nm) and aminoacyl nucleolipid **14** (~460 nm). Alternatively, the complex formed between DNA and **14** illustrates a greater than two fold increase in particle sizes from 0 to 36 h, suggesting the formation of a higher-order complex structure.

In order to assess the anti-cancer activity of aminoacyl nucleolipid **14**, a cancer cell line screening assay was conducted against a panel of 60 cell lines selected at the National Cancer Institute (NCI).[23] A single dose (10 μM) screen was performed with **14**,

Table 2
Effective diameters (nm) of aminoacyl nucleolipid, **14**, and DNA+**14** at 25 °C

Compound	Time (h)	Effective diameters (nm)
14	0	468 ± 50
	36	400 ± 50
DNA+14	0	980 ± 50
		4500 ± 100
	36	9300 ± 100

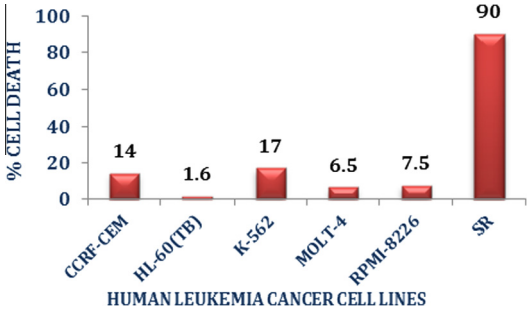


Figure 5. Cell death of human leukemia cell lines following treatment with **14** (10 μM) for 48 h.

and cell viability was determined following 48 h incubation with cancer cells at 37 °C. A sulforhodamine B (SRB cell viability assay)[24] was performed to determine cell death in the presence of **14**. Aminoacyl nucleolipid **14** was found to exhibit potent and selective anti-cancer activity (see Supplementary data, Fig. S3), with greater than 90% cell death against a SR human lymphoblastic leukemia cancer cell line (Fig. 5). Interestingly, GRP78 expression levels are elevated in leukemic blasts of adult patients and early relapse in childhood leukemia[25] suggesting a correlation between the GRP78 DNA binding affinity and anti-leukemic activity of aminoacyl nucleolipid **14**. We are currently investigating the bio-molecular basis for the potent and selective activity of **14** in leukemia cells.

In conclusion, with the synthesis of derivative **14**, we have demonstrated the utility of a new class of aminoacyl nucleolipid, which can have great relevance due to their tight binding with GRP78 DNA and marked ability to trigger potent and selective anti-leukemic activity. This work paves the way for additional studies into the mechanism of aminoacyl nucleolipid anti-cancer activity. Moreover, **14** may form the basis for novel ligand design and development for anti-cancer applications.

Acknowledgments

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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.2013.07.030.

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