Bioorganic & Medicinal Chemistry Letters 20 (2010) 3150–3154

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Macrocyclic hexaoxazoles: Influence of aminoalkyl substituents on RNA and DNA G-quadruplex stabilization and cytotoxicity

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ARTICLE INFO

Article history:
Received 28 January 2010
Revised 19 March 2010
Accepted 26 March 2010
Available online 30 March 2010

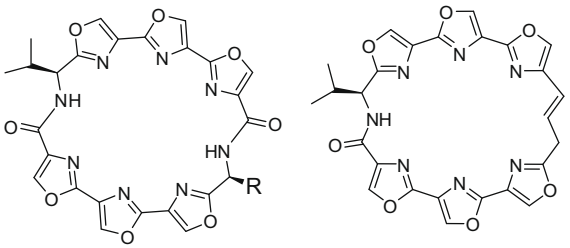
Keywords:
G-quadruplex DNA
G-quadruplex RNA
Macrocycles
Hexaoxazoles
Antitumor
MDA-MB-435 xenograft
Cytotoxicity
Athymic nude mice
G-quadruplex ligands

ABSTRACT

A series of 24-membered macrocyclic hexaoxazoles containing one or two aminoalkyl substituents was synthesized and evaluated for cytotoxicity and for their ability to selectively stabilize G-quadruplex DNA and RNA. The most cytotoxic analog **4a**, with IC_{50} values of 25 and 130 nM using KB3-1 and RPMI 8402 cells, is efficacious in vivo in athymic nude mice with a human tumor xenograft from the breast cancer cell line MDA-MB-435.

Nucleic acid sequences that can form stable G-quadruplexes have been implicated in a wide range of biological functions. G-quadruplexes are associated with the regulation of telomere stability, regulatory elements in promoters, and viral integration and recombination.[1–3] Several research groups have developed molecules that are focused on targeting and stabilizing G-quadruplex DNA as anticancer agents. Selective G-quadruplex interactive ligands have the potential to act as cancer-specific agents by inhibiting the overexpression of oncogenes such as c-Myc, c-Kit, and KRAS or blocking the 3′ extension of telomeric DNA by telomerase.[1,2] Telomestatin is a macrocyclic polyoxazole isolated from *Streptomyces anulatus* 3533-SV4 that can stabilize G-quadruplex DNA in a highly selective manner.[4] Our laboratory has synthesized several 24-membered macrocyclic polyoxazoles that are highly selective for G-quadruplexes.[5–7] We have recently reported on the biophysical and pharmacologic properties of these G-quadruplex stabilizers.[8–10]

HXDV (Fig. 1), a 24-membered macrocycle containing six oxazole moieties and two valine residues, is cytotoxic toward human lymphoblastoma RPMI 8402 cells with an IC_{50} value of 0.4 μM.[5] HXPV (Fig. 1), a 24-membered macrocycle formed by ring-closing metathesis, has an IC_{50} of 25 nM.[6] Neither HXDV or HXPV have physicochemical properties that permit facile formulation for assessment of their potential efficacy as antitumor agents in vivo. Lysinyl macrocyclic hexaoxazole analogs with improved properties for formulation were synthesized and identified as selective G-quadruplex interactive ligands.[7] These results were subsequently confirmed by another laboratory.[11] Recently, a lysinyl macrocyclic



HXDV; R = $CH(CH_3)_2$
LV; R = $(CH_2)_3CH_2NH_2$

HXPV

Figure 1. Structures of HXDV, HXPV, and HXLV.

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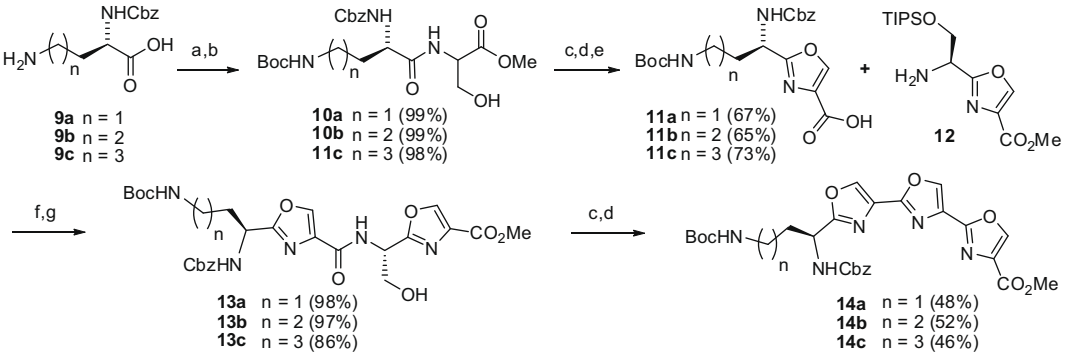
doi:10.1016/j.bmcl.2010.03.086

heptaoxazole with similar cytotoxicity (IC_{50} = 670 nM) to HXDV has also been reported.[12] The present study examines the influence of alkylamino chain length within a series of 24-membered hexaoxazoles on cytotoxicity with the objective of selecting a suitable macrocyclic hexaoxazole for in vivo evaluation of antitumor activity in athymic nude mice with the breast cancer cell line MDA-MB-435 as the human tumor xenograft.

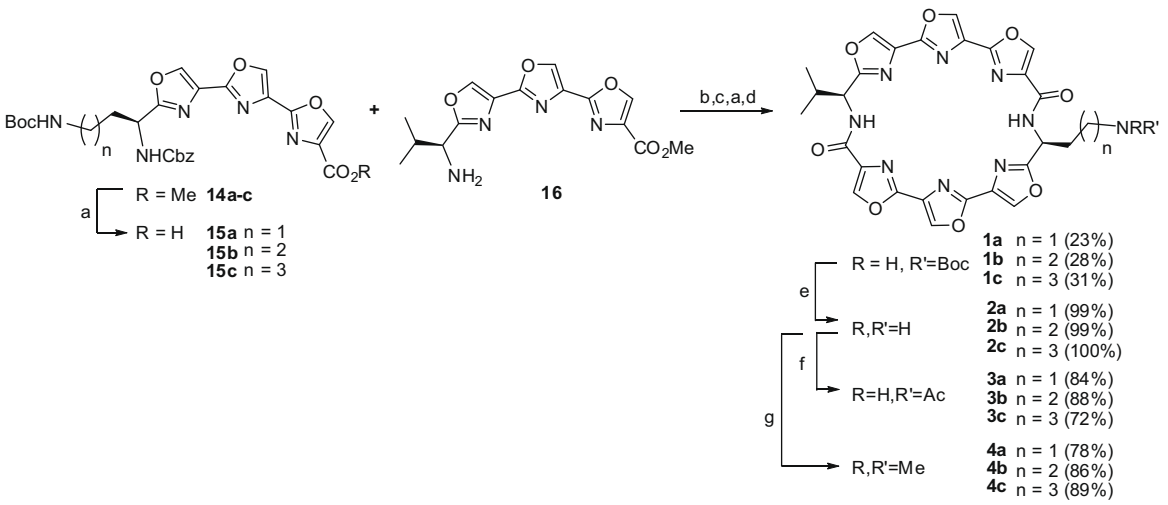
The synthetic approach used in the preparation of the eighteen new aminoalkyl substituted hexaoxazole macrocycles listed was similar to that previously reported for HXLV.[7a] The three *N*-Boc aminoalkyl teroxazoles **14a–c** were synthesized as outlined in Scheme 1. Hydrolysis of the methyl ester of each of these aminoalkyl teroxazoles provided the carboxylic acid derivative, **15a–c**, which were each then subsequently condensed as illustrated in Scheme 2 with the valine teroxazole **16**, prepared as previously described.[5] Macrocyclization of the hexaoxazole intermediate was accomplished by hydrogenolysis of the *N*-Cbz group, hydrolysis of the methyl ester, and lactam formation using HATU and *N*-methylmorpholine (NMM).

The bis-aminoalkyl substituted hexaoxazole macrocycles were prepared as outlined in Scheme 3. The *N*-Cbz group was removed from **14a–c** by hydrogenolysis to provide the primary amine derivatives **17a–c**, which were condensed with the carboxylic acid derivatives **15a–c**. The remaining *N*-Cbz group of the resulting hexaoxazole was removed by hydrogenolysis and the methyl ester hydrolyzed with LiOH in THF. Formation of the macrocyclic lactam was accomplished using either BOPCl, DMAP, DIPEA, DMF for **5a** and **5b** or BOP, HOBt, DIPEA, NaI, DMF for **5c**. Primary amines **2a–c** and **6a–c** were prepared by treatment of the Boc derivatives with TFA. Acetylation provided acetamide derivatives **3a–c** and **7a–c**. Reductive methylation of **2a–c** and **6a–c** afforded the *N*,*N*-dimethylamine derivatives, **4a–c** and **8a–c**.

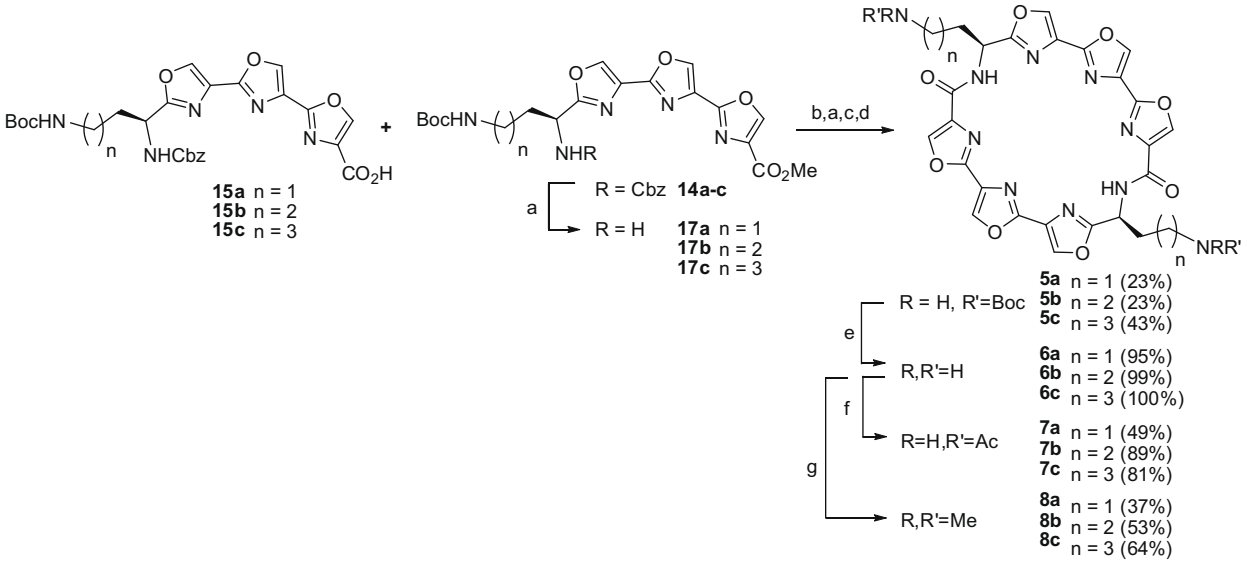
The cytotoxic activities of these various aminoalkyl substituted macrocyclic hexaoxazoles are summarized in Table 1. The *N*-Boc and bis-*N*-Boc derivatives of these aminoalkyl hexaoxazoles, **1a–c** and **5a–c**, have similar cytotoxic activities towards RPMI 8402, with IC_{50} values ranging from 0.29 to 1.40 μM. Similar activity was observed with the human carcinoma cell line KB3-1 with IC_{50} values ranging from 0.23 to 0.45 μM. The primary amine derivatives of substituted hexaoxazoles with a single aminoalkyl side chain, **2a–c**, were less cytotoxic than their *N*-Boc derivatives, but were more cytotoxic than their bis-aminoalkyl derivatives **6a–c** in both of these cell lines. Similarly, the *N*-acetyl derivatives of the hexaoxazole analogs with a single aminoalkyl side chain **3a–c** were also more cytotoxic than the bis-*N*-acetyl derivatives, **7a–c**. In comparing the cytotoxicity of the *N*,*N*-dimethylaminoalkyl derivative **4a–c** with the bis-*N*,*N*-dimethylaminoalkyl derivatives **8a–c**, it is evident that the presence of a second *N*,*N*-dimethylaminoalkyl substituent is associated with a dramatic loss in activity. Among the bis-dimethylaminoethyl analogs, **8a** is the most cytotoxic with IC_{50} values of 4.3 and 2.8 μM in RPMI 8402 and KB3-1 cells, respectively. The most striking difference in cytotoxicity observed among homologs was



Scheme 1. Synthesis of aminoalkyl teroxazoles. Reagents and conditions: (a) Boc_2O, $NaHCO_3$; (b) Serine Me ester HCl, BOP, Et_3N; (c) DAST, K_2CO_3; (d) DBU, $BrCCl_3$; (e) LiOH, THF/H_2O; (f) EDC, HOBt, 2,6-lutidine; (g) HF/pyridine.



Scheme 2. Synthesis of HXDV derivatives with an aminoalkyl chain. Reagents and conditions: (a) LiOH, THF/H_2O; (b) EDC, HOBt, 2,6-lutidine; (c) H_2(g), 20% $Pd(OH)_2$/C, THF/MeOH for **15a** and **15b**, 1,4-cyclohexadiene, 20% $Pd(OH)_2$/C, D, THF/EtOH for **15c**; (d) HATU, NMM, DMF; (e) TFA, CH_2Cl_2; (f) Ac_2O, pyridine; (g) MeOH, HCl, HCHO/H_2O, $NaBH_4$.



Scheme 3. Synthesis of HXDV derivatives with two aminoalkyl chains. Reagents and conditions: (a) H_2(g), 20% $Pd(OH)_2$/C, THF/EtOH for **14a** and **14b**, 1,4-cyclohexadiene, 20% $Pd(OH)_2$/C, Δ, THF/EtOH for **14c**; (b) EDC, HOBt, 2,6-lutidine; (c) LiOH, THF/H_2O; (d) BOPCl, DMAP, DIPEA, DMF for **5a** and **5b**, BOP, HOBt, DIPEA, NaI, DMF for **5c**; (e) TFA, CH_2Cl_2; (f) Ac_2O, pyridine; (g) MeOH, HCl, HCHO/H_2O, $NaBH_4$.

Table 1
Relative cytotoxic activities. IC_{50} values (μM)[a]

Compound	RPMI 8402	KB3-1 wt	KBV-1 + MDR1	KBH5.0 + BCRP
1a	0.31 ± 0.13	0.29 ± 0.09	>10	0.25 ± 0.07
1b	0.29 ± 0.01	0.23 ± 0.04	>10	0.15 ± 0.07
1c	0.35 ± 0.07	0.3 ± 0.00	>10	0.33 ± 0.11
2a	1.2 ± 0.28	2.2 ± 1.1	>10	1.2 ± 1.1
2b	2.5 ± 0.71	0.8 ± 0.57	>10	0.38 ± 0.04
2c	4.8 ± 1.7	3.4 ± 2.5	>10	3.0 ± 1.4
3a	0.95 ± 0.07	0.45 ± 0.07	>10	0.90 ± 0.14
3b	2.5 ± 0.71	3.4±	>10	3.0 ± 0.00
3c	0.75 ± 0.21	0.71 ± 0.38	>10	1.9 ± 1.3
4a	0.13 ± 0. 08	0.025 ± 0.007	7.5 ± 3.5	0.018 ± 0.011
4b	0.23 ± 0.04	0.30 ± 0.14	>10	0.07 ± 0.04
4c	>10	3.2 ± 1.8	3.2 ± 1.8.	2.7 ± 0.21
5a	0.35 ± 0.07	0.25 ± 0.07	>10	0.25 ± 0.07
5b	1.4 ± 0.85	0.45 ± 0.21	>10	0.38 ± 0.04
5c	0.40 ± .07	0.25 ± 0.7	>10	0.33 ± 0.11
6a	>10	>10	>10	>10
6b	>10	6.3 ± 3.2	>10	3.1 ± 0.84
6c	n.d.	>10	>10	>10
7a	>10	>10	>10	>10
7b	>10	>10	>10	>10
7c	3.3 ± 2.1	4.0 ± 1.7	>10	4.5 ± 2.1
8a	4.3 ± 2.5	2.8 ± 0.35	>10	0.65 ± 0.35
8b	>10	4.2 ± 1.2	>10	3.0 ± 0.00
8c	>10	4.0 ± 2.6	>10	3.6 ± 2.0
HXPV	0.03 ± 0.01	0.04 ± 0.01	3.5 ± 0.67	0.04 ± 0.02
HXDV	0.54 ± 0.12	0.35 ± 0.08	7.7 ± 2.1	0.45 ± 0.19

[a] Values are means of three experiments ± standard deviation, n.d. = not determined.

with those hexaoxazoles with a single dimethylaminoalkyl moiety, **4a**–**c**. In RPMI 8402, the IC_{50} values ranged from 0.13 to 11 μM. The dimethylaminoethyl derivative **4a**,[13] the shortest homolog, is the most cytotoxic. This clear difference in cytotoxic potency among homologs was also observed in KB3-1 cells, with IC_{50} values of 0.025–3.2 μM, with **4a** again being the most cytotoxic. The cytotoxicity of **4a** is substantially greater than HXDV and is comparable to HXPV, which has previously been recognized as being among the most cytotoxic of these oxazole-containing macrocyclic G-quadruplex selective ligands. KBV-1 and KBH5.0 cells are variants of KB1-1 that overexpress the efflux transporters MDR1 and BCRP, respectively. The data summarized in Table 1 indicate that all of the macrocyclic hexaoxazoles with IC_{50} values less than 5.0 μM in KB3-1 cells are resistant in KBV-1 cells and are likely MDR1 substrates. In contrast, no notable resistance was observed with these hexaoxazoles in KBH5.0 cells, with the exception of **4c**, suggesting that these analogs are not substrates for the BCRP efflux transporter.

The in vivo efficacy of **4a** as an antitumor agent was evaluated in athymic nude mice with human tumor xenografts developed from implanted MDA-MB-435 breast tumor cells.[14] This assay was performed as previously described by our laboratory.[15] The positive control group consisted of mice (8 mice per group) injected ip 3× weekly with irinotecan at a dose of 20 mg/kg for 31 days. The vehicle control group consisted of seven mice that received ip injections 3× weekly of 150 μl of 10 mM citrate for 31 days. Compound **4a** was administered by ip injection 3× weekly at a concentration of 4.0 mg per mL in 10 mM citrate at doses of 14, 18, 31 mg/kg for weeks 1–3, respectively. For the final 10 days mice received a dose of 42 mg/kg 3× per week for an average dose of 28.8 mg/kg. On day 35, the average tumor volume ± SE of the control group was 1396 ± 296 mm^3, compared to 85 ± 29 mm^3 for mice treated with irinotecan and 302 ± 27 mm^3 for mice treated with **4a**.

Four selected macrocyclic hexaoxazoles (HXDV, **4a**, **4b**, and **4c**) were evaluated for their ability to selectively bind and stabilize the quadruplex versus duplex forms of DNA and RNA in the presence of physiological concentrations (150 mM) of K^+ ions. We used salmon testes (ST) DNA and poly(rA)·poly(rU) as representative models of duplex DNA and RNA, respectively. As a representative model of quadruplex DNA, we used the human telomeric sequence d[$T_2G_3(T_2AG_3)_3$A], which we denote as hTel. Patel and co-workers have shown that the structure adopted by d[$T_2G_3(T_2AG_3)_3$A] in K^+ solution is an intramolecular (3 + 1) G-quadruplex in which three strands are oriented in one direction and the fourth strand is oriented in the opposite direction.[16] In an effort to explore ligand binding interactions with quadruplex RNA, we used a putative quadruplex-forming sequence (r[AG_4CG_2-$CUG_2UCG_2AGUG_2C$]) derived from the 5′-untranslated region of the mRNA that encodes the cell-cycle checkpoint protein kinase Aurora A (AurA). We selected this RNA sequence for the present studies in light of our recently reported observations that HXDV treatment of cancer cells

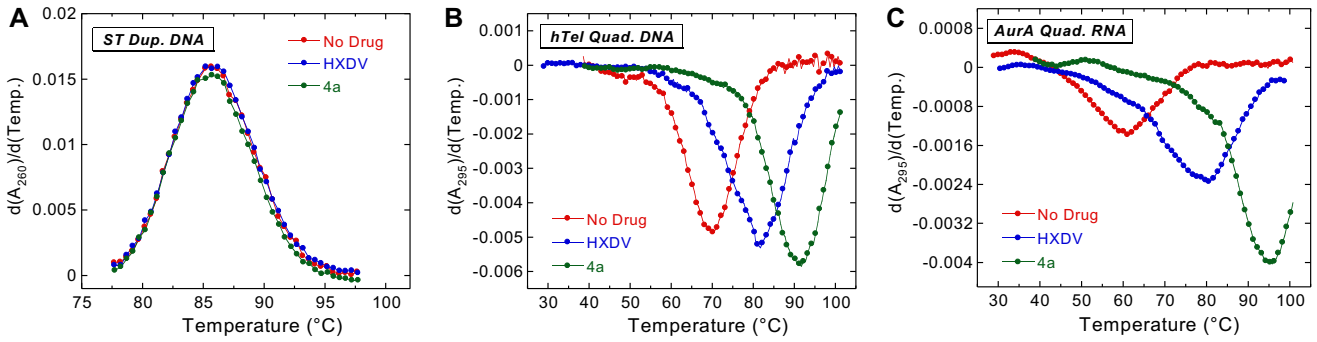


Figure 2. First derivatives of the UV melting profiles of ST duplex DNA (A), hTel quadruplex DNA (B), and AurA quadruplex RNA (C) in the absence (red) and presence of either HXDV (blue) or **4a** (green). The UV melting profiles of the quadruplexes were acquired at 295 nm, while those of the duplexes were acquired at 260 nm. In the quadruplex melting studies, the hTel and AurA concentrations were 5 μM in strand. When present in these quadruplex studies, the drug concentrations were 20 μM. In the duplex melting studies, the ST DNA concentrations were 30 μM base pair. When present, the drug concentrations were 15 μM in the ST DNA studies. In all experiments, the solution conditions were 10 mM potassium phosphate (pH 7.5) and sufficient KCl (132 mM) to bring the total K^+ concentration to 150 mM.

Table 2
Impact of macrocyclic hexaoxazoles on the thermal stabilities of quadruplex DNA and RNA

Compound	ΔT_{tran}[a] (°C)	
	hTel Quadruplex DNA	AurA Quadruplex RNA
HXDV	11.5	19.5
4a	21.5	34.5
4b	24.0	ND
4c	2.0	ND

[a] ΔT_{tran} reflects the change in transition temperature (T_{tran}) of the target nucleic acid induced by the presence of the compound. Values of T_{tran} were determined from the maxima or minima of first-derivative UV melting profiles exemplified by those shown in Figure 2B and C. The uncertainty in the ΔT_{tran} values is ±0.5 °C. ND denotes not determined.

induces down-regulation of AurA protein levels and mitotic arrest.[10] These results raise the intriguing possibility that macrocyclic hexaoxazoles may bind and stabilize a quadruplex structure in the mRNA sequence of AurA, thereby interfering with the translation of the mRNA.

HXDV, **4a**, **4b**, and **4c** exert negligible impacts on the thermal stabilities of either the ST DNA duplex (Fig. 2A and not shown for **4a**–**c**) or the poly(rA)·poly(rU) RNA duplex (not shown). This observation is consistent with little or no duplex DNA or RNA binding on the part of any of the four macrocyclic hexaoxazoles tested here. We had observed previously a similar behavior for HXDV.[5–8] In marked contrast to their negligible impacts on the thermal stabilities of the DNA and RNA host duplexes, HXDV, **4a**, **4b**, and **4c** increase the T_{tran} value of the hTel DNA quadruplex by 11.5, 21.5, 24.0, and 2.0 °C, respectively (Fig. 2B and Table 2).

These results are not only indicative of binding to hTel, but also provide information with regard to the relative affinities of the compounds for the host DNA quadruplex. In this connection, the relative extents to which ligands stabilize a target nucleic acid are typically correlated with the relative binding affinities of the ligands for the target.[17,18] Our hTel UV melting results are therefore consistent with the affinities of HXDV, **4a**, **4b**, and **4c** for the target DNA quadruplex following the hierarchy **4b** > **4a** > HXDV > **4c**. Thus, replacement of a valine linker in HXDV with an *N*,*N*-dimethylaminoalkyl functionality appears to enhance affinity for the hTel DNA quadruplex, but only when the alkyl group is an ethyl (**4a**) or a propyl (**4b**). When the alkyl group is a butyl (**4c**), however, affinity for the hTel DNA quadruplex appears to be decreased. Figure 2C shows the UV melting profiles of the AurA RNA sequence in the absence and presence of HXDV and **4a**.

The temperature-induced hypochromic transition at 295 nm exhibited by the AurA RNA sequence in the absence of ligand is consistent with this RNA oligomer adopting a quadruplex structure in the presence of 150 mM K^+. In support of this observation, circular dichroism (CD) studies suggest that the AurA sequence forms a parallel-stranded RNA structure (see Supplementary Fig. S1). Note that the presence of HXDV and **4a** enhances the thermal stability of the AurA RNA quadruplex by 19.5 and 34.5 °C, respectively (Table 2). These results are indicative of ligand-induced stabilization of a quadruplex structure in the AurA target RNA sequence. In the aggregate, our collective UV melting studies indicate that macrocyclic hexaoxazoles bind the quadruplex nucleic acid form with a high degree of specificity. It should also be noted that the relative extents to which HXDV, **4a**, **4b**, and **4c** stabilize the quadruplex nucleic acid form correlate reasonably well with their relative cytotoxic potencies (see Table 1). This observation supports the hypothesis that a quadruplex structure (RNA or DNA) could serve as a principle cytotoxic target of the macrocyclic hexaoxazoles. Similar results have recently been observed with macrocyclic pyridyl polyoxazoles that have been developed as G-quadruplex ligands.[19]

Acknowledgment

These studies were supported in part by the Department of Defense Grant W81XWH06-1-0514.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.03.086.

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