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Quinone Methides Tethered to Naphthalene Diimides as Selective G-Quadruplex Alkylating Agents

Marco Di Antonio,[†] Filippo Doria,[‡] Sara N. Richter,[§] Carolina Bertipaglia,[†] Mariella Mella,[‡] Claudia Sissi,[†] Manlio Palumbo,*[,†] and Mauro Freccero*[,‡]

Dipartimento di Chimica Organica, Università di Pavia, V.le Taramelli 10, 27100 Pavia, Italy, Dipartimento di Scienze Farmaceutiche, Università di Padova, Via Marzolo, 5, 35131 Padova, Italy, and Dipartimento di Istologia, Microbiologia e Biotecnologie Mediche, Università di Padova, Via Gabelli, 63, 35121, Padova, Italy

Received June 15, 2009; E-mail: mauro.freccero@unipv.it; manlio.palumbo@unipd.it

Abstract: We have developed novel G-quadruplex (G-4) ligand/alkylating hybrid structures, tethering the naphthalene diimide moiety to quaternary ammonium salts of Mannich bases, as quinone-methide precursors, activatable by mild thermal digestion (40 °C). The bis-substituted naphthalene diimides were efficiently synthesized, and their reactivity as activatable bis-alkylating agents was investigated in the presence of thiols and amines in aqueous buffered solutions. The electrophilic intermediate, quinone-methide, involved in the alkylation process was trapped, in the presence of ethyl vinyl ether, in a hetero Diels−Alder [4 + 2] cycloaddition reaction, yielding a substituted 2-ethoxychroman. The DNA recognition and alkylation properties of these new derivatives were investigated by gel electrophoresis, circular dichroism, and enzymatic assays. The alkylation process occurred preferentially on the G-4 structure in comparison to other DNA conformations. By dissecting reversible recognition and alkylation events, we found that the reversible process is a prerequisite to DNA alkylation, which in turn reinforces the G-quadruplex structural rearrangement.

Introduction

Guanine-rich regions, such as the 5′-TTAGGG repetitive sequence contained in the human telomeres, can form tetraplex assemblies known as G-quadruplexes (G-4).[1] A recent approach to telomerase inhibition is based on the conformational remodeling of the DNA repetitive telomeric structures, which involves sequestering its substrate, the single-stranded telomeric DNA, by inducing it to fold into G-4 structure(s).[2] Stabilization of G-4 conformations has been achieved by using a great variety of small molecules, acting as G-4 selective ligands.[3] Because of their recognition properties toward nucleic acids, derivatives of 1,8-naphthalimide, such as 1,4,5,8-naphthalene tetracarboxylic diimides (NDIs) and perylene analogues (PDIs) have been extensively studied in recent decades and shown to act by intercalation or end-stacking.[4] In more detail, a few naphthalene imides and diimides including dimeric derivatives bind selectively duplex DNA.[5] Several naphthalimide-containing com-

[†] Dipartimento di Scienze Farmaceutiche, Università di Padova.
[‡] Dipartimento di Chimica Organica, Università di Pavia.
[§] Dipartimento di Istologia, Microbiologia e Biotecnologie Mediche, Università di Padova.

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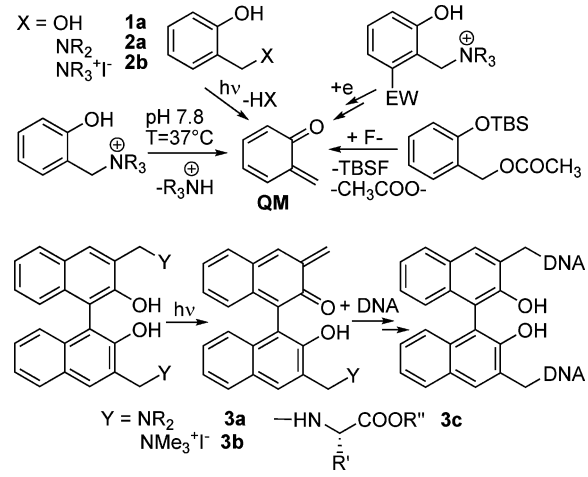
pounds have also been described as photoinduced DNA cleaving agents,[6] and some of them have been entered into clinical trials owing to their strong anticancer activity.[7] Only very recently, a series of disubstituted NDIs have also been screened as G-4 ligands, showing lower affinity in comparison to the more extended perylene analogues.[8] More promising G-4 binding properties have been achieved by Neidle's group using tri- and tetra-substituted NDIs.[9]

Very recently, covalent modifications and, more generally, chemical reactions have been suggested as effective strategies for developing methods aimed at probing G-4 structures.[10] Possible G-4 alkylating species thus far reported include Pt-complexes[11] and quaternary ammonium porphyrins.[12] As a matter of fact, the first example refers to coordination of the metal ion to a purine N7 in the tetraplex structure (G-quadruplex platination),[13] and the second infers the possibility of photoinduced G-4 cross-linking, by a carbon electrophile (alkylation), considering that phenol quaternary ammonium derivatives can cross-link duplex DNA efficiently upon irradiation.[14]

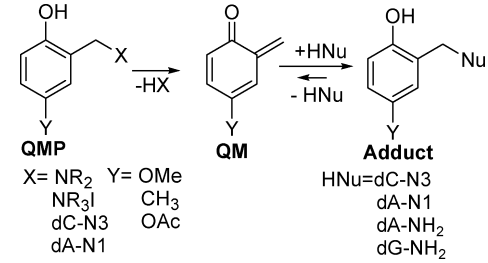
Chemical reactions to be exploited for G-4 recognition and covalent modification should be very mild, selective, and readily initiated. To achieve this aim with single organic compounds, quinone methide (QM) reactivity has been exploited. QMs are transient Michael acceptors which can be generated from suitable and hydrosoluble precursors by mild activation.[14]

Several groups, including our own, have shown that *o*-hydroxy benzyl alcohols (**1**),[15] Mannich base derivatives of phenols (**2**)[16,17] and binols (**3**),[14b,c] are capable of undergoing mono- and bis-alkylation in water and DNA cross-linking by photoactivation,[14b] mild base catalysis,[14c] thermal digestion under physiological conditions or in the presence of fluoride anion,[18] and mild monoelectronic reduction (Scheme 1).[19]

Scheme 1. Generation of QMs from Phenol (**1a**, **2a,b**) and Binol (**3a**−**c**) Derivatives



Scheme 2. Substituent Effects on the Reactivity of QM-Precursors (QMP)



When studying a model QM in the presence of biological nucleophiles (including deoxynucleosides), formation of QMs was demonstrated to depend upon the leaving group at the benzylic position of its precursor (QMP, in Scheme 2).[18] Furthermore, both the generation of QMs under physiological conditions and the thermodynamic stability of the resulting adducts were shown to be highly responsive to both the presence of electron-withdrawing and -donating substituents on the aromatic ring (Y, in Scheme 2)[20] and on the nature of the leaving group (X, in Scheme 2).

Electron-donating groups, including a *p*-alkyl substituent, facilitate QM generation, under mild conditions (Scheme 2, Y = CH_3), while electron-withdrawing groups strongly suppress QM formation, making the resulting adducts more stable.[20,21] In more detail, quaternary ammonium salts of Mannich bases

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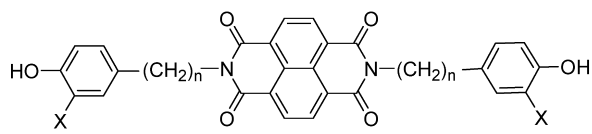
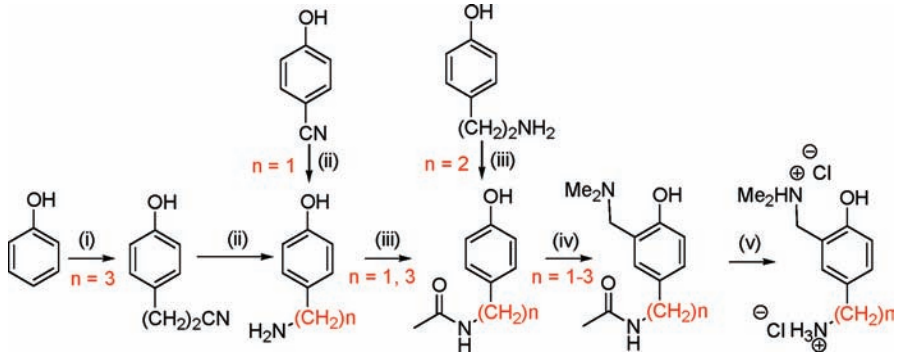
Chart 1. Bis-Substituted NDIs **4**−**6** Investigated as G-4 Ligand-Alkylating Hybrid Compounds



n= 1, X= CH_2NMe_3I **4**
CH_2NMe_2 **4a**
n= 2, X= CH_2NMe_3I **5**
CH_2NMe_2 **5a**
H **5b**
n= 3, X= CH_2NMe_3I **6**
CH_2NMe_2 **6a**

Scheme 3. Synthesis of 4-Aminomethyl-, 4-(2-Aminoethyl)-, and 4-(3-Aminopropyl)-2-dimethylaminomethylphenols (n = 1, 2, and 3, respectively)[a]



[a] Reagents and conditions: (i) acrylonitrile as solvent, $AlCl_3$; (ii) $LiAlH_4$, THF, Δ, 4 h; (iii) Ac_2O, $NaHCO_3$, H_2O, rt; (iv) paraformaldehyde, dimethylamine in anhydrous EtOH, Δ, 2 h; (v) HCl aq 10%, 2 h, Δ.

($X = NR_3^+$) bearing a methoxy and a methyl group ($Y = OCH_3$, CH_3) have been shown to act both as QM-precursors (QMPs) under physiological conditions (25 °C < T < 40 °C, 6.0 < pH ≤ 7.8), but only the latter generate fairly stable alkylation adducts.[20] When using nucleosides as nucleophiles, only the conjugate adducts with deoxycytidine at the N3 (dC-N3), deoxyadenosine at both N1 and NH_2 (dA-N1 and dA-NH_2), and deoxyguanosine at NH_2 (dG-NH_2) are stable in water solution. Contrary, the alkylation adducts at N7 of both dG and dA are thermodynamically unstable, since the reversal of the alkylation becomes a competitive process (Scheme 2).[22]

Therefore, taking advantage of such a latent alkylating reactivity and the G-4 recognizing properties of the NDI moiety, we decided to investigate the activation conditions and the reactivity and the G-4 binding properties of three new bis-alkylating QMPs tethered to the NDI core, by conformationally flexible $(CH_2)_n$ spacers (n = 1−3; **4**−**6**, Chart 1). The aim of this work is to demonstrate that the G-4 binding properties of fairly weak G-4 reversible ligands, such as disubstituted NDIs, can be strengthened by alkylation of the target, using two QMP moieties as activatable alkylating agents. These compounds clearly differ from the reported Pt-complexes[11,13] as the alkylation step by a QM needs activation to be performed, which in principle allows spatial and temporal control of the reaction process. In addition a large number of QM-generating species can be devised, which again allows modulation in reactivity and hence in the conditions at which alkylation is performed. This can be particularly relevant in view of therapeutic applications of the QMP-NDI conjugates.

Results and Discussion

Synthesis of Naphthalene Diimides Tethered to Quinone-methide Precursors. The quaternary ammonium salts **4**−**6** used as ligand-alkylating hybrid compounds were prepared by exhaustive methylation of the diamines **4a**−**6a** (Chart 1). The NDIs **4a**−**6a** were synthesized in a one-pot synthesis from the commercially available 1,4,5,8-naphthalenetetracarboxylic acid dianhydride and 4-aminomethyl-, 4-(2-aminoethyl)-, and 4-(3-aminopropyl)-2-dimethylaminomethylphenols, respectively. The above intermediates have been prepared according to the general synthetic procedure depicted in Scheme 3 and described in detail in Supporting Information.

Activation and Reactivity of Naphthalene Diimides 4−**6.** Our study began with the investigation of the reactivity of the NDIs **4**−**6**, in order to define the most convenient protocol for their activation as bis-alkylating agents. We performed digestion of **4**−**6** at both 25 and 40 °C in the presence of several simple nucleophiles (thiols and amines), in aqueous buffered and unbuffered solutions. The results are collected in the Table 1.

Activation by base catalysis in the presence of amines under unbuffered conditions takes advantage of the low pK_a (∼7.8) of the quaternary ammonium salts of the three NDIs **4**−**6**, which in the presence of basic nucleophiles allows the generation of reactive zwitterionic forms of **4**−**6** at pH ≥ 8. The latter promptly release the alkylating QM (Scheme 1) at 25 °C. In the presence of nonbasic nucleophiles such as thiols, a longer reaction time (6−12 h) is required at 40 °C under neutral conditions. Specific base catalysis in buffered solutions at pH 8.5 triggered the alkylation of the less basic nucleophiles at lower temperature (25 °C), affording the alkylated adducts in good yields (≥90%).

In the absence of a nucleophile, hydration reaction occurred, affording diols **7**, **14**, and **17** in low yield at 25 °C. Higher yields in a shorter reaction time were achieved at 40 °C. Data in Table 1 suggest that the NDIs **4**−**6** exhibit a very similar reactivity, since they can be activated as bis-alkylating agents under the very same mild conditions (pH 7, at 40 °C, for less than 12 h).

Amines **4a**−**6a**, unlike their quaternary ammonium salts **4**−**6**, are stable for at least 24 h at 40 °C at both pH 7.0 and 8.5. Therefore, they cannot generate alkylating QMs under these mild conditions.

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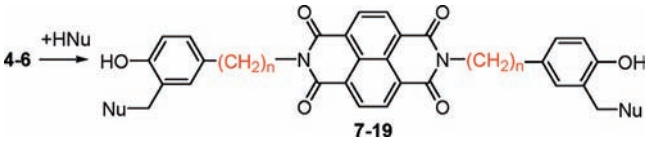
Table 1. Reactivity of **4**−**6** in the Presence of Nucleophiles



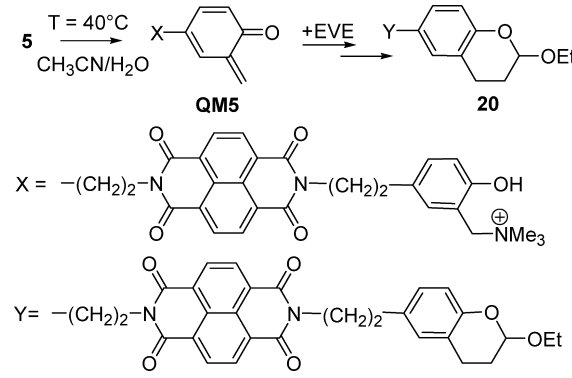
Adduct	n	Nu	Conditions, (Yield %)[a]
7	2	HO-	12h; 40°C; (25) [c]
			2h; 25°C; (20) [d]
8	2	Et_2N-	0.5h; 40°C; (95)[b]
9	2		0.5h; 40°C; (97)[b]
10	2		0.5h; 40°C; (85)[b]
11	2		0.5h; 40°C; (90) [b]
			2h; 25°C; (86) [d]
12	2	*t*-BuNH-	6h; 40°C; (88) [b]
13	2	*t*-BuS-	12h; 40°C; (96) [b,c]
			2h; 25°C; (90) [d]
14	1	HO-	12h; 40°C; (27) [c]
			2h; 25°C; (25) [d]
15	1	Et_2N-	0.5h; 40°C; (97)[b]
16	1	*t*-BuS-	12h; 40°C; (98) [b,c]
			2h; 25°C; (92) [d]
17	3	HO-	12h; 40°C; (30) [c]
			2h; 25°C; (32) [d]
18	3	Et_2N-	0.5h; 40°C; (98)[b]
19	3	*t*-BuS-	12h; 40°C; (95) [b,c]
			2h; 25°C; (94) [d]

[a] Reaction time and temperature; CH_3CN/H_2O = 1:1, [**4**−**6**] = 10^{-3}M, [HNu] = 10^{-2} M. [b] Unbuffered. [c] Buffered at pH 7.0. [d] Buffered at pH 8.5.

To unequivocally clarify the nature of the electrophilic intermediate involved in the alkylation process, we ran additional trapping experiments in the presence of EVE (ethyl vinyl ether), in buffered aqueous acetonitrile (1:1). Compound **5** gave **20** (58%, yield) as the main product in the presence of the hydration adduct **7** (38%). The formation of the substituted 2-ethoxychroman **20** is only possible through the intermediacy of two 1,4-dipolar species trapped by EVE in hetero-Diels−Alder [4 + 2] cycloaddition reactions (Scheme 4). Therefore, the efficient generation of **20** in aqueous acetonitrile provides conclusive evidence for the involvement of two sequential quinone methides such as QM**5**.

Alkylation of DNA Folded into a G-Quadruplex. The initial assessment of the ability of compounds **4**−**6** to alkylate DNA

Scheme 4. Generation and Reactivity of QM**5** (Tethered to the NDI Moiety) as Heterodiene



in a G-4 conformation was achieved by reacting increasing concentrations (0.25−16 μM) of the compounds at 40 °C for 24 h in buffer Li_3PO_4 10 mM, KCl 50 mM, pH 7.4, with a ^{32}P 5′-end labeled synthetic oligonucleotide formed by four human telomeric repeats, 5′-AGGGTTAGGGTTAGGGTTAGGG-3′ (4GGG). In these conditions, 4GGG can fold into an intramolecular G-4 preferentially assuming a hybrid-type (mixed parallel/antiparallel) conformation.[23] The alkylated oligo was separated from the nonreacted DNA by denaturing polyacrylamide gel. As shown in Figure 1A, compound **5** exhibited alkylating properties. The covalent adduct was detectable at a concentration as low as 0.25 μM (lane 2, Figure 1A) and reached 15% over total DNA at compound **5** concentration of 2 μM (lane 5, Figure 1A). At higher ligand concentrations no further increment in the alkylated adduct was observed. This is likely related to the NDI−DNA complex precipitation (lanes 6−8, Figure 1A) occurring at high molar ratios. Reactivity of **5** lead to maximal 4GGG adduct formation in 24 h at 40 °C whereas alkylation was substantially absent at 20 °C. In addition, the identified alkylation product was stable upon incubation at 20−70 °C. Degradation was appreciable at higher temperatures (Figure 1S, Supporting. Information).

When the reactivity of NDIs with different linkers was monitored, compound **4** was able to alkylate only 5% of the total DNA at the highest tested concentration (16 μM), whereas compound **6** gave no appreciable adduct with 4GGG (Figure 1C). These data indicate that n = 2 is the best linker length among bis-substituted NDIs. Additionally, compound **5a** (amine analogue of **5**) did not form any alkylation product, confirming that only the quaternary ammonium salt derivative is active in the test conditions (Figure 1C).

Drug Alkylation as a Function of DNA Folding. Selective recognition of G-quadruplex DNA by **5** was assessed by comparing its reactivity toward 4GGG with (i) single-stranded DNA exhibiting the same base composition of 4GGG DNA but unable to fold into a G-4 conformation (ss-scrambled-4GGG) and (ii) ss-scrambled-4GGG annealed to its complementary oligo (ds-scrambled-4GGG) (Figure 1A). Double-stranded DNA turned out to be the poorest substrate for **5** adduct formation. Interestingly, comparison of reactivity toward G-4 folded and ss-scrambled oligos showed a concentration window where alkylation occurs more efficiently on the G-4 folded DNA (Figure 1B). Indeed, at concentration below 1 μM, **5** was able to form a covalent adduct with 4GGG DNA only (lanes 2−4,

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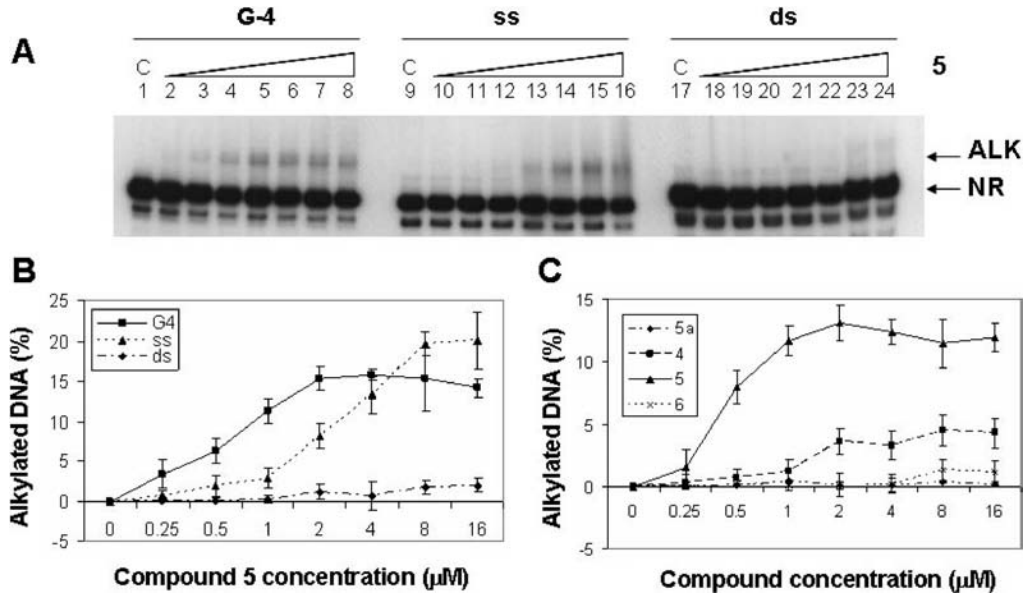

Figure 1. Specificity of NDI-mediated alkylation. (A) Increasing concentrations of compound **5** (0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 μM) were incubated with labeled 4GGG (G-4), ss-scrambled-4GGG (ss), or ds-scrambled-4GGG (ds) DNA for 24 h at 40 °C in Li_3PO_4 10 mM, KCl 50 mM, pH 7.4. Alkylated DNA (ALK) was separated from nonreacted oligo (NR) in 20% denaturing polyacrylamide gel. "C" refers to labeled 4GGG DNA without compound **5**. (B) Quantification of the specificity of the alkylation experiment reported in panel A. (C) Quantification of alkylation of G-4 DNA by compounds **5a**, **4**, **5**, **6**. Increasing compound concentrations (0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 μM) were reacted with 4GGG DNA for 24 h at 40 °C in Li_3PO4 10 mM, KCl 50 mM, pH 7.4. Adducts (ALK) were separated from nonreacted DNA (NR) by 20% denaturing polyacrylamide gel.

Figure 1A and B). Conversely, ss-stranded 4GGG was alkylated at higher concentrations (2−16 μM) (lanes 13−16, Figure 1A and B). This clearly suggests a modulation of drug reactivity upon DNA folding.

To evaluate if this behavior derives from a selective drug−DNA interaction, competition experiments were performed. Thus, a constant concentration of compound **5** was incubated with a fixed amount of ^{32}P 5′-end labeled 4GGG DNA in the presence of increasing concentrations of cold 4GGG, ss-scrambled-4GGG, or ds-scrambled-4GGG DNA. As shown in Figure 2, cold 4GGG DNA was able to effectively compete for adduct formation (adduct amounts decreased to less than 2% of total labeled DNA in the presence of a 10-fold excess of 4GGG DNA competitor) (lane 5, Figure 2A and B). On the contrary, the alkylated adduct was only modestly affected with ss and ds-scrambled-4GGG competitor DNAs (Figure 2). These data clearly demonstrate that compound **5** can specifically alkylate G-4 conformed DNA.

The apparent selectivity of compounds **5** toward differently conformed DNAs depends on both reversible and irreversible target recognition. Whereas experiments below (see G-quadruplex folding induction versus alkylation chapter) clarify their interdependency, different techniques other than electrophoretic methods are warranted to define reversible binding constants (K_d) and the orientation of the electrophilic center with respect to the alkylation site.

G-Quadruplex Folding Induction versus Alkylation. To clarify how this selectivity is related to the DNA recognition step, we used a ^{32}P 5′-end labeled synthetic oligonucleotide containing only two repeats of the human telomeric sequence, 5′-TACAGATAGTTAGGGTTAGGGTTA-3′ (2GGG). This oligo can fold into G-4 arrangements only by intermolecular pairing leading to dimeric or tetrameric species resolvable by native gel electrophoresis. This process is poorly efficient in the absence of a G-4 stabilizer, thus allowing us to compare

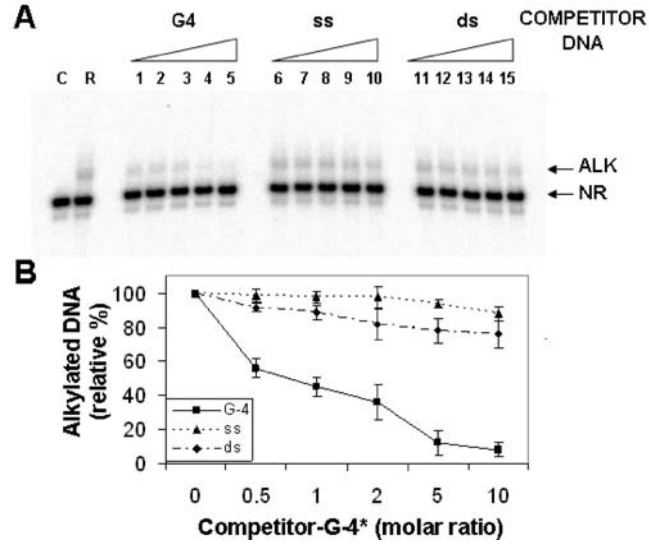

Figure 2. Competition of NDI-mediated alkylation. (A) Compound **5** (2 μM) was incubated with labeled 4GGG (3.9 pmol) in the presence of increasing molar ratios (0.5, 1, 2, 5, 10) of cold 4GGG (G-4), ss-scrambled-4GGG (ss), or ds-scrambled-4GGG (ds) DNA for 24 h at 40 °C. Alkylated DNA (ALK) was separated from nonreacted oligo (NR) in 20% denaturing gel. "C" refers to labeled 4GGG (3.9 pmol) treated at 40 °C for 24 h, and "R" refers to the 4GGG-compound **5** reaction product in the absence of any competitor DNA. (B) Quantification of the competition experiment in panel A. Adduct amount obtained in sample R was taken as 100%, and quantification of adduct amount in lanes 1−15 was calculated as relative percent.

the ability of compound **5** to induce G-4 folding and alkylation on the same DNA sequence. DNA−ligand incubation was performed using alkylating (40 °C) and nonalkylating (20 °C) conditions in the presence/absence of 100 mM K^+, to promote G-4 conformation. At variable time, samples were loaded onto

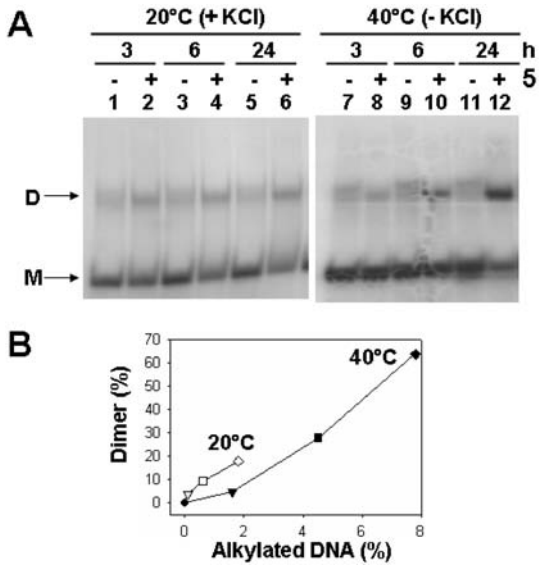

Figure 3. Comparison of induction of G-4 conformation and alkylation by NDI **5**. 2GGG DNA was incubated in the presence (+) or absence (−) of compound **5** (G-4/**5** molar ratio = 0.04) for variable times at 20 or 40 °C. (A) The monomeric DNA (M) was separated from the dimeric G-4 DNA (D) by 16% native polyacrylamide gel. (B) Quantification data. Dimer formation is reported as a function of DNA alkylation. Values were taken at 0 (circles), 3 (rectangles), 6 (squares), and 24 (diamonds) h time intervals after incubation at 20 °C (open symbols) or 40 °C (filled symbols). Dimer formation and DNA alkylation were calculated as percent over total DNA obtained as integration values of electrophoretic bands from native (A) and denaturing (not shown) gels, respectively.

16% native and 20% denaturing polyacrylamide gels, to check for folding stabilization and alkylation, respectively.

According to previous results with NDI derivatives,[8] using reversible conditions, **5** was poorly effective in inducing G-4 structures. Indeed, incubation of 2GGG with 25 μM **5** at 20 °C induced only 18% of G-4 dimer at 24 h (lanes 1−6, Figure 3A). In these incubation conditions, the presence of KCl was demanding; indeed, at 20 °C no quadruplex stabilization was observed in the absence of KCl. Correspondingly, negligible alkylation occurred at this incubating temperature (data not shown).

Remarkably, the G-4 folded dimeric conformation was induced by **5** upon incubation at 40 °C (lanes 7−12, Figure 3A). Denaturing gel showed that in these conditions 2GGG alkylation products were detectable after 6 and 24 h of incubation (not shown). Direct comparison of G-4 folding induction and alkylation indicates that the amount of DNA adduct is linearly related to the extent of G-4 folding. However, since compound **5** induces a larger amount of G-4 structure than it undergoes alkylation, we infer that G-4 folded DNA stabilization occurs prior to and is a prerequisite for DNA alkylation, which in turn irreversibly stabilizes the NDI-G-4 complex.

CD Spectroscopic Study. The above conclusion is supported by an analysis of the chiroptical properties of 4GGG alone and in the presence of **5** (Figure 4). In our experimental conditions, 4GGG preferentially folds into a mixed type G-4 structure.[1a,24,25] Upon 24 h incubation with **5** at 20 °C, hence in the absence of alkylation events, modest changes occurred in the DNA dichroic spectra that represent the sum of DNA folding rearrangements and ligand-induced CD. It is interesting to note that after 24 h of incubation at 40 °C, a larger modulation of the dichroic response occurred. In particular, a significant increment of the 265 nm band associated with a reduction of the 295 nm one emerged. This suggests that DNA can undergo a conformational rearrangement in the presence of **5**. In line with the gel shift data, the modest level of alkylation obtained in these conditions should not justify the extensive conformational change presented in Figure 4. Hence, reversible interaction between derivative **5** and G-4 represents the main responsible for the observed conformational changes.

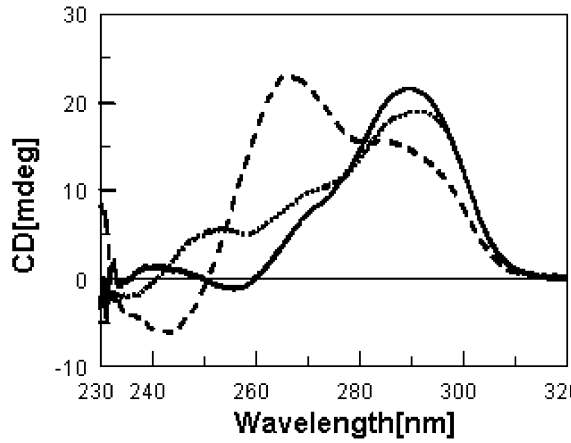

Figure 4. CD spectra of 4 μM 4GGG incubated for 24 h alone (solid line) and in the presence of 26 μM **5** at 25 °C (dotted line) or 40 °C (dashed line) in Li_3PO_4 10 mM, KCl 50 mM, pH 7.4.

Alkylated G-Quadruplex Properties. With the aim to identify a potential selective alkylation site along the G-quadruplex folded 4GGG, we used a exonuclease I assay.[26] Adduct instability at temperatures above 70 °C prevented us from using standard techniques, such as Taq polymerase stop assay or thermal depurination at alkylated sites. The ^{32}P 5′-end labeled alkylated G-4 DNA was gel purified and treated with increasing amounts of exonuclease I at 50 °C for 30 min, alongside the nonalkylated G-4 DNA treated in the same conditions. As shown in Figure 5, the enzyme could not process the alkylated G-4 DNA even at the highest tested enzyme amounts, whereas it was able to digest the nonalkylated oligo, with an efficiency linearly related to enzyme concentrations.

The fact that no enzyme stop positions were observed likely reflects a lack of DNA-enzyme recognition. On the basis of the data obtained by circular dichroism studies, this behavior is probably related to the structural rearrangement occurring on the 4GGG bound by compound **5**, which prevents anchoring/processing by exonuclease I. However, we cannot exclude a 3′-end DNA alkylation event which would result in a similar experimental outcome.

Stabilization of G-4 conformation upon compound **5** alkylation was further supported by CD melting analysis, according

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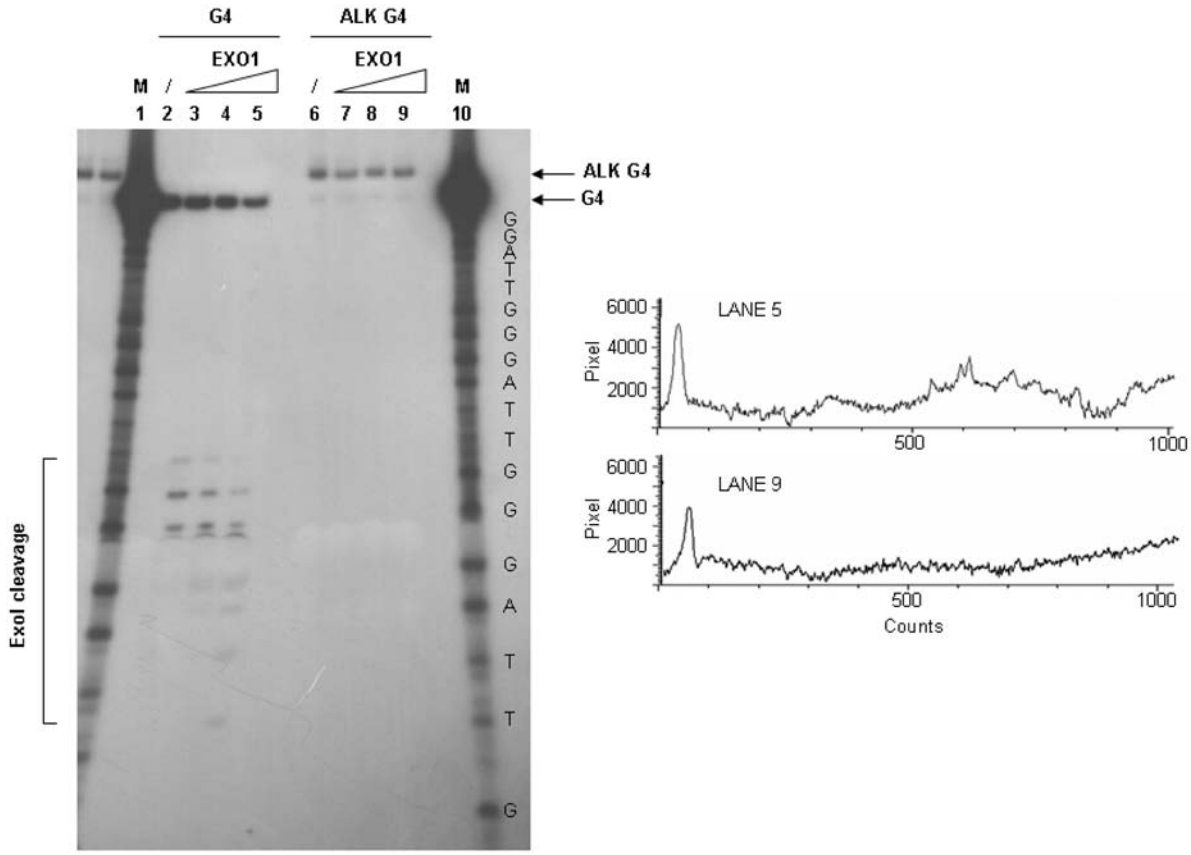

Figure 5. Digestion of alkylated and nonalkylated G-4 DNA by exonuclease I. Labeled 4GGG DNA (G4) and the purified labeled **5**-alkylated 4GGG DNA (ALK G4) were incubated with increasing amounts of Exonuclease I (ExoI) (10, 20, and 40 units) for 30 min at 50 °C. Cleavage products were analyzed in 20% denaturing polyacrylamide gel. "M" indicates purine markers run aside samples. Densitometry profiles referring to lanes 5 and 9 are shown on the right.

to which a T_m above 75 °C was observed (data not shown). As already mentioned, however, above 70 °C the alkylated adduct is not stable (see Figure 1S, Supporting Information), and hence the observed T_m value reflects a number of processes (i.e., G-4 folding/unfolding, reversible NDI association/dissociation to/from DNA, and covalent bond formation/disruption) which take place concurrently at high temperatures.

NDIs Cytotoxicity. Cytotoxic effects of NDI derivatives **4**, **5**, and **6** were investigated using the human embryonic kidney 293T cell line. Cells were exposed to increasing concentrations of the tested compounds (12 nM−40 μM) at 37 °C for 48 h, after which cell damage was assessed by MTT assay. The effective drug concentration able to kill 50% of cell population after drug exposure (EC_{50}) was 4.5 ± 0.3 μM, 10.5 ± 3.5 μM, and >40 μM for compounds **5**, **4**, and **6**, respectively. Compound **5b**, which was also included as a control ligand that completely lacks the ability to generate QM, displayed EC_{50} > 40 μM. These data indicate that the cytotoxic potency of these compounds depends on and parallels their DNA alkylating ability.

Conclusion

In summary, we have described the first example of thermally induced non-metal-based G-quadruplex adduct formation, where a reversible binding process is associated with selective alkylation and stabilization of the G-4. This goal has been achieved using novel ligand/alkylating hybrid structures exhibiting a NDI core tethered to activatable alkylating moieties by flexible spacers. We have shown that this strategy is effective in the enhancement of the G-4 folding induction and stabilization, appearing to be a promising tool for probing G-4 structures.

Experimental Section

***N,N′*-Bis(3-dimethylaminomethyl-4-hydroxybenzyl)-1,4,5,8-naphthalenetetracarboxylic Acid Diimide (4a).** A 3.2 g portion of 4-aminomethyl-2-dimethylaminomethyl phenol dihydrochloride (12.6 mmol) and 1.61 g (6.0 mmol) of 1,4,5,8 tetracarboxylic dianhydride were suspended in 20 mL of a mixture dioxane/DMF 9:1. Under nitrogen atmosphere, 1 mL of TEA was added to the suspension, and the mixture was allowed to reflux with vigorously stirring for 5 h. The reaction mixture was cooled at room temperature and poured into 30 mL of water. The resulting suspension was filtered and washed with water and cold EtOH anhydrous. The **4a** was obtained as a pale yellow solid. Yield 91%. Mp > 350 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 8.80 (s, 4H), 7.40 (d, 2H, J = 6.9 Hz), 7.20 (s, 2H), 6.85 (d, 2H, J = 6.9 Hz), 5.20 (s, 4H), 3.60 (s, 4H), 2.30 (s, 12H). ^{13}C NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 162.74; 157.76; 131.03; 130.95; 131.00; 129.55; 126.87; 126.61; 121.68; 115.91; 62.60; 44.37; 43.41. Anal. Calcd for $C_{34}H_{32}N_4O_6$: C, 68.91; H, 5.44; N, 9.45; O, 16.20. Found: C, 68.95; H, 5.40; N, 9.47.

4. A 1.7 g (2.9 mmol) portion of the bisimide **4a** was suspended in 50 mL of CH_3CN and 1.2 g (8.5 mmol) of CH_3I was added. This suspension was refluxed under nitrogen atmosphere and in a few minutes the reaction mixture turned dark red. After heating 3 h at 80 °C, the reaction was cooled at room temperature. Red solid formation was observed after addition of Et_2O (50 mL) to

the reaction mixture. The suspension was filtered and washed with CH_3CN to give 2.7 g (3.0 mmol) of **4** as a red-brownish solid. Mp > 350 °C. Yield 92%. ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C, TMS): δ 10.30 (s, 2H), 8.70 (s, 4H), 7.50 (s, 2H), 7.40 (d, 2H, J = 8.4 Hz), 6.90 (d, 2H, J = 8.4 Hz), 5.15 (s, 4H), 4.45 (s, 4H), 3.05 (s, 18H). ^{13}C NMR (DMSO-d_6, 25 °C, TMS): 162.72; 156.59; 134.88; 132.07; 130.71; 127.63; 126.38; 126.28; 116.09; 114.39; 62.91; 51.90; 42.7. Anal. Calcd for $C_{36}H_{38}I_2N_4O_6$: C, 49.33; H, 4.37; I, 28.96; N, 6.39; O, 10.95. Found: C, 49.35; H, 4.41; I, 28.93; N, 6.42.

***N*,*N*′-Bis[2-(3-dimethylaminomethyl-4-hydroxyphenyl)-ethyl]-1,4,5,8-naphthalenetetracarboxylic Acid Diimide (5a).** The same protocol used for the synthesis of **4a** was followed; 2.1 g of *N*-(3-((dimethylamino)methyl)-4-hydroxyphenylethyl)amine dihydrochloride (8.3 mmol) and 1.07 g (4.0 mmol) of 1,4,5,8 tetracarboxylic dianhydride were used. The **5a** was obtained as a pale yellow solid, 2.20 g (yield 89%). Mp > 350 °C ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 8.70 (s, 4H), 7.20 (d, 2H, J = 7.4 Hz), 7.00 (s, 2H), 6.80 (d, 2H, J = 7.4 Hz), 4.40 (t, 4H, J = 8.1 Hz), 3.75 (s, 4H), 2.90 (t, 4H, J = 8.1 Hz), 2.35 (s, 12H). ^{13}C NMR (DMSO-d_6, 25 °C, TMS): δ 162.46; 155.08; 132.92; 131.29; 130.48; 129.00; 126.27; 126.12; 116.34; 115.71; 54.54; 45.15; 32.43; 30.78. Anal. Calcd for $C_{36}H_{36}N_4O_6$: C, 69.66; H, 5.85; N, 9.03; O, 15.47. Found: C, 69.62; H, 5.88; N, 9.00.

5. The same protocol used for the synthesis of **4** was followed; 2.0 g (3.2 mmol) of the bisimide **5a** was used and 2.7 g (3.0 mmol) of **5** as a red-brownish solid was obtained. Mp > 350 °C. Yield 84%. ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C, TMS): δ 10.20 (s, 2H), 8.80 (s, 4H), 7.30 (s, 2H), 7.20 (d, 2H, J = 7.6 Hz), 6.90 (d, 2H, J = 7.6 Hz), 4.40 (s, 4H), 4.20 (t, 4H, J = 8.1 Hz), 3.00 (s, 18H), 2.85 (t, 4H, J = 8.1 Hz). ^{13}C NMR (DMSO-d_6, 25 °C, TMS): δ 162.50; 155.80; 134.72; 132.38; 130.47; 129.28; 126.30; 126.14; 116.15; 114.63; 63.06; 51.95; 41.60; 32.41. Anal. Calcd for $C_{38}H_{42}I_2N_4O_6$: C, 50.46; H, 4.68; I, 28.06; N, 6.19; O, 10.61. Found: C, 50.41; H, 4.71; I, 28.01; N, 6.16.

***N*,*N*′-Bis[2-(4-hydroxyphenyl)ethyl]-1,4,5,8-naphthalene-tetracarboxylic Acid Diimide (5b).** The same protocol used for the synthesis of **4a** was followed; 1.0 g of Tyramine (7.3 mmol) and 0.84 g (3.0 mmol) of 1,4,5,8-tetracarboxylic dianhydride was used. The **5b** was obtained as a yellow solid, 1.50 g (yield 99%). Mp > 350 °C. ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C, TMS) δ 9.20 (s, 2H), 8.70 (s, 4H), 7.10 (d, 4H, J = 7.9 Hz), 6.80 (d, 2H, J = 7.9 Hz), 4.20 (t, 4H, J = 8.1 Hz), 2.80 (s, 4H). ^{13}C NMR (DMSO-d_6, 25 °C, TMS): δ 162.63; 156.62; 130.55; 129.51; 127.15; 126.31; 115.05; 45.35; 32.01. Anal. Calcd for $C_{30}H_{22}N_2O_6$ C, 71.14; H, 4.38; N, 5.53; O, 18.95 Found: C, 71.55; H, 4.58; N, 5.31.

***N*,*N*′-Bis[3-(3-dimethylaminomethyl-4-hydroxyphenyl) propyl]-1,4,5,8-naphthalenetetracarboxylic Acid Diimide (6a).** The same protocol used for the synthesis of **4a** was followed; 1.17 g of 4-(3-aminopropyl)-2-dimethylaminomethyl phenol dihydrochloride (6.01 mmol) and 0.8 g (3.0 mmol) of 1,4,5,8 tetracarboxylic dianhydride were used. The **6a** was obtained as a pale yellow solid, 2.2 g (yield 98%). Mp > 350 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 8.75 (s, 4H), 7.35 (d, 2H, J = 7.6 Hz), 6.85 (s, 2H), 6.70 (d, 2H, J = 7.6 Hz), 4.25 (t, 4H, J = 6.0 Hz), 3.60 (s, 4H), 2.69 (t, 4H, J = 6.0 Hz), 2.30 (s,12H), 2.10 (t, 4H, J = 8.0 Hz). ^{13}C NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 162.70; 155.99; 130.77; 128.22; 127.93; 126.48; 121.61; 126.12; 115.71; 62.74; 44.38; 40.70; 32.37; 29.44. Anal. Calcd for $C_{38}H_{40}N_4O_6$: C, 70.35; H, 6.21; N, 8.64; O, 14.80 Found: C, 70.37; H, 6.22; N, 8.86.

6. The same protocol used for the synthesis of **4** was followed; 0.90 g (1.4 mmol) of the bisimide **6a** was used and 1.06 g of **6** as a red-brownish solid was obtained. Mp > 350 °C. Yield 81%. ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C, TMS): δ 10.15 (s, 2H), 8.60 (s, 4H), 7.25 (m, 4H), 6.85 (d, 2H, J = 8.6 Hz), 4.40 (t, 4H), 4.05 (s, 4H), 3.05 (s, 18H), 2.65 (t, 4H); 1.95 (t, 4H). ^{13}C NMR (DMSO-d_6, 25 °C, TMS): 163.07; 157.35; 134.82; 132.60; 130.53; 126.97; 126.63; 126.34; 116.43; 114.84; 62.81; 52.04. Anal. Calcd for $C_{40}H_{46}I_2N_4O_6$: C, 51.51; H, 4.97; I, 27.21; N, 6.01; O, 10.29 Found: C, 51.46; H, 4.93; I, 27.19; N, 6.06

Activation of the Bis-alkylating Properties by Mild Thermal Digestion. General Procedures. Base Catalysis General Method. To trap the nucleophiles by mild thermal digestion the following procedures were used:

Procedure A (for basic nucleophiles: pK_a > 8). To 50 mL of a solution 5×10^{-3} M of the bisimides (**4**−**6**) in 1:1 CH_3CN/H_2O, outgassed with a N_2 flux, was added the nucleophile (5×10^{-2} M). This solution is allowed to stand at 40 °C and after 0.5 h a pale yellow precipitate starts to form. The reaction mixture was kept at 40 °C for 4 h. After this time the CH_3CN was evaporated under vacuum, and the suspension was cooled at 4 °C for 12 h. The bis-alkylated adduct formed was filtered and washed with cold anhydrous EtOH to provide the product in good yield (60−85%).

Procedure B (for non basic nucleophiles: pK_b < 8). To 50 mL of a solution 5×10^{-3} M of the bisimides (**4**−**6**) in 1:1 CH_3CN/H_2O buffered to pH 8.5 with a $H_2PO_4^-/HPO_4^{2-}$ buffer, outgassed with a N_2 flux, was added the nucleophile (5×10^{-2} M). This solution was heated at 40 °C for 12 h. After this time the CH_3CN was evaporated under vacuum and the suspension was kept at 4 °C for 12 h. The bis-alkylated adduct precipitated was filtered and washed with cold water and anhydrous EtOH to provide the product in fairly good yield (55−70%)

Adduct 7. Pale yellow needles. Mp > 350 °C. ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C, TMS): δ 10.20 (s, 2H), 8.70 (s, 4H), 7.30 (s, 2H), 7.20 (d, 2H, J = 7.9 Hz), 6.90 (d, 2H, J = 7.9 Hz), 4.30 (s, 4H), 4.20 (t, 4H); 2.9 (t, 4H).^{13}C NMR (DMSO-d_6, 25 °C, TMS): δ 162.48; 155.80; 134.71; 132.37; 130.47; 129.30; 126.28; 126.13; 116.16; 114.63; 63.11; 51.99; 41.60; 32.41. Anal. Calcd for $C_{32}H_{26}N_2O_8$: C, 67.84; H, 4.63; N, 4.94; O, 22.59. Found: C, 67.81; H, 4.66; N, 4.95.

Adduct 8. Yellow needles. Mp > 350 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 8.80 (s, 4H), 7.10 (d, 2H, J = 8.1 Hz), 7.00 (s, 2H), 6.75 (d, 2H, J = 8.1 Hz), 4.40 (t, 4H, J = 7.8 Hz), 3.75 (s, 4H); 2.95 (t, 4H, J = 7.8 Hz); 2.60 (q, 8H, J = 7.1 Hz); 1.10 (t, 12H, J = 7.1 Hz). ^{13}C NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 162.58; 156.88; 132.90; 130.82; 128.78; 128.21; 126.52; 126.42; 122.11; 115.93; 56.80; 46.18; 42.38; 33.24; 11.12. Anal. Calcd for $C_{40}H_{44}N_4O_6$: C, 70.99; H, 6.55; N, 8.28; O, 14.18. Found: C, 71.03; H, 6.51; N, 8.33.

Adduct 9. Pale yellow needles. Mp > 350 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 8.90 (s, 4H), 7.15 (d, 2H, J = 7.9 Hz), 7.00 (s, 2H), 6.90 (d, 2H, J = 7.9 Hz), 4.40 (t, 4H, J = 7.4 Hz), 3.75 (s, 4H); 2.95 (t, 4H, J = 7.4 Hz); 2.60 (m, 8H); 1.50 (q, 4H, J = 6.5 Hz); 1.25 (q, 4H, J = 6.1 Hz); 1.1 (t, 6H, J = 6.1 Hz); 0.90 (t, 6H, J = 6.5 Hz). ^{13}C NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 162.58; 156.79; 130.83; 128.78; 128.20; 126.52; 122.17; 115.88; 57.39; 52.49; 46.57; 44.38; 42.40; 33.26; 28.54; 20.46; 13.86; 10.84. Anal. Calcd for $C_{44}H_{52}N_4O_6$: C, 72.11; H, 7.15; N, 7.64; O, 13.10. Found: C, 72.13; H, 7.11; N, 7.68.

Adduct 10. Yellow needles. Mp > 350 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS):δ 8.80 (s, 4H), 7.10 (d, 2H, J = 7.5 Hz), 7.00 (s, 2H), 6.75 (d, 2H, J = 7.5 Hz), 4.45 (t, 4H, J = 7.4 Hz), 3.60 (t, 8H, J = 7.1 Hz); 3.00 (t, 8H, J = 7.1 Hz), 2.90 (t, 4H, J = 7.4 Hz). ^{13}C NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 163.12; 156.34; 130.36; 129.72; 128.47; 126.98; 126.20; 122.79; 122.17; 115.88; 66.13; 58.01; 52.49; 46.57; 33.26. Anal. Calcd for $C_{40}H_{40}N_4O_8$: C, 68.17; H, 5.72; N, 7.95; O, 18.16. Found: C, 68.13; H, 5.70; N, 7.91.

Adduct 11. Yellow needles. Mp > 350 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 8.6 (s, 4H); 7.20 (d, 2H, J = 7.7 Hz); 7.00 (s, 2H); 6.75 (d, 2H, J = 7.7 Hz); 4.45 (t, 4H, J = 7.8 Hz); 3.60 (s, 4H); 3.0 (t, 4H, J = 7.8 Hz); 2.4 (m, 8H); 1.8 (m, 8H).^{13}C NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 163.56; 156.60; 130.82; 128.84; 128.28; 128.17; 126.57; 126.50; 122.45; 15.80; 58.66; 53.35; 42.34; 33.22; 23.53. Anal. Calcd for $C_{40}H_{40}N_4O_6$: C, 71.41; H, 5.99; N, 8.33; O, 14.27. Found: C, 71.38; H, 6.04; N, 8.30.

Adduct 12. Green crystals. Mp > 350 °C: ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 8.80 (s, 4H); 7.10 (d, 2H, J = 7.8 Hz), 7.00 (s, 2H), 6.85 (d, 2H, J = 7.8 Hz); 4.40 (t, 4H, J = 7.7 Hz); 4.00 (s, 4H); 2.90(t, 4H, J = 7.7 Hz); 1.20 (s, 18H). ^{13}C NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 162.58; 157.03; 130.85; 128.81; 128.39; 128.34; 126.53; 123.54; 116.43; 50.98; 45.95; 42.45; 33.29; 28.47. Anal. Calcd for $C_{40}H_{44}N_4O_6$:C, 70.99; H, 6.55; N, 8.28; O, 14.18. Found: C, 70.92; H, 6.58; N, 8.22.

Adduct 13. Yellow needles. Mp > 350 °C. ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C, TMS): δ 10.20 (s, 2H), 8.80 (s, 4H); 7.20 (d, 2H, J = 7.9 Hz), 7.0 (s, 2H), 6.85 (d, 2H, J = 7.9 Hz); 4.45 (s, 4H), 4.30 (t, 4H, J = 7.6 Hz); 3.15 (s, 18H), 3.00 (t, 4H, J = 7.6 Hz). ^{13}C NMR (DMSO-d_6, 25 °C, TMS): δ 162.79; 157.12; 130.82; 128.81; 128.39; 128.34; 126.93; 126.51; 123.54; 116.43; 60.21; 45.95; 42.41; 38.29; 33.47 Anal. Calcd for $C_{40}H_{42}N_2O_6S_2$: C, 67.58; H, 5.95; N, 3.94; O, 13.50; S, 9.02. Found: C, 67.51; H, 5.98; N, 3.92; S, 9.00

Adduct 14. Yellow needles. Mp > 350 °C. ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C, TMS): δ 10.00 (s, 2H), 8.70 (s, 4H), 7.30(s, 2H), 7.20 (d, 2H, J = 7.8 Hz), 6.90 (d, 2H, J = 7.8 Hz), 5.1 (s, 4H), 4.30 (s, 4H). ^{13}C NMR (DMSO-d_6, 25 °C, TMS): δ 162.48; 155.80; 134.71; 132.37; 130.47; 129.30; 126.28; 126.13; 121.16; 115.93; 63.11; 62.58. Anal. Calcd for $C_{30}H_{22}N_2O_8$: C, 66.91; H, 4.12; N, 5.20; O, 23.77. Found: C, 67.01; H, 4.17; N, 5.26.

Adduct 15. Yellow needles. Mp > 350 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 8.70 (s, 4H), 7.30 (s, 2H), 7.00 (d, 2H, J = 7.6 Hz), 6.90 (d, 2H, J = 7.6 Hz), 5.1 (s, 4H), 4.20 (s, 4H) 2.60 (q, 8H, J = 7.1 Hz); 1.1 (t, 12H, J = 7.1 Hz). ^{13}C NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 163.70; 155.99; 131.29; 130.77; 128.22; 127.93; 126.48; 121.61; 115.71; 62.74; 44.38; 40.70; 32.37; 29.44. Anal. Calcd for $C_{38}H_{40}N_4O_6$: C, 70.35; H, 6.21; N, 8.64; O, 14.80. Found: C, 70.33; H, 6.27; N, 8.62.

Adduct 16. Yellow needles. Mp > 350 °C. ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C, TMS): δ 10.80 (s, 2H), 8.80 (s, 4H), 7.50(s, 2H), 7.40 (d, 2H, J = 8.8 Hz), 7.20 (d, 2H, J = 8.8 Hz), 5.1 (s, 4H), 4.30 (s, 4H), 3.00(s, 18H). ^{13}C NMR (DMSO-d_6, 25 °C, TMS): δ 163.44; 155.98; 132.55; 131.59; 130.99; 126.96; 126.72; 126.21; 116.02; 115.76; 62.18; 60.26; 45.78; 38.30. Anal. Calcd for $C_{38}H_{38}N_2O_6S_2$: C, 66.84; H, 5.61; N, 4.10; O, 14.06; S, 9.39. Found: C, 66.81; H, 5.55; N, 4.06; S, 9.34.

Adduct 17. Yellow needles. Mp > 350 °C. ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C, TMS): δ 10.15 (s, 2H), 8.60 (s, 4H), 7.25 (d, 2H, J = 8.0 Hz), 7.0 (s, 2H); 6.85 (d, 2H, J = 8.0 Hz), 4.40 (t, 2H, J = 7.5 Hz); 4.30 (s, 4H); 2.65 (t, 2H, J = 7.5 Hz); 2.00 (m, 4H). ^{13}C NMR (DMSO-d_6, 25 °C, TMS): δ 163.23; 158.35; 134.82; 132.60; 130.53; 126.97; 126.63; 126.34; 116.43; 114.84; 62.81; 52.04; 33.25; 29.07. Anal. Calcd for $C_{34}H_{30}N_2O_8$:C, 68.68; H, 5.09; N, 4.71; O, 21.53. Found: C, 68.71; H, 5.11; N, 4.74.

Adduct 18. Yellow needles. Mp > 350 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 8.70 (s, 4H), 7.10 (d, 2H, J = 7.9 Hz), 7.00 (s, 2H), 6.90 (d, 2H, J = 7.9 Hz), 4.40 (t, 4H, J = 7.6 Hz), 3.70 (s, 4H); 2.95 (t, 4H, J = 7.6 Hz); 2.60 (q, 8H, J = 7.1 Hz); 2,10 (t, 4H, J = 7.6 Hz) 1.1 (t, 12H, J = 7.1 Hz). ^{13}C NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 162.17; 155.17; 132.91; 130.45; 128.79; 128.31; 126.55; 126.42; 122.09; 116.13; 57.39; 46.57; 44.38; 33.26; 28.54; 20.46. Anal. Calcd for: $C_{42}H_{48}N_4O_6$ C, 71.57; H, 6.86; N, 7.95; O, 13.62. Found: C, 71.52; H, 6.88; N, 7.89.

Adduct 19. Yellow needles. Mp > 350 °C. ^{1}H NMR (300 MHz, DMSO-d_6, 25 °C, TMS): δ 10.10 (s, 2H), 8.70 (s, 4H), 7.00 (d, 2H, J = 7.7 Hz), 6.90 (s, 2H), 6.90 (d, 2H, J = 7.7 Hz), 4.25 (t, 4H, J = 6.0 Hz), 3,60 (s, 4H), 2.69 (t, 4H, J = 6.0 Hz), 2.00 (s, 18H), 2.10 (t, 4H, J = 8.0 Hz). ^{13}C NMR (DMSO-d_6, 25 °C, TMS): δ 163.04; 156.42; 132.46; 131.43; 130.56; 126.92; 126.62; 126.31; 117.75; 115.99; 62.90; 61.46; 32.37; 29.44; 22.47; 18.34. Anal. Calcd for $C_{42}H_{46}N_2O_6S_2$: C, 68.27; H, 6.27; N, 3.79; O, 12.99; S, 8.68. Found: C, 68.22; H, 6.21; N, 3.82; S, 8.69.

Adduct 20. Yellow needles. Mp > 350 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 8.70 (s, 4H), 7.20 (d, 2H, J = 8.2 Hz), 7.10 (s, 2H), 6.75 (d, 2H, J = 8.2 Hz), 5.25 (dd, 2H, J = 2.7 Hz), 4.40 (t, 4H, J = 8.0 Hz), 3.90 (m, 2H), 3.60 (m, 2H), 3.00 (t, 4H, J = 8.0 Hz), 2.60 (m, 2H), 2.00 (m, 2H), 1.30 (t, 6H, J = 7.0 Hz). ^{13}C NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 162.59; 150.8; 130.85; 130.10; 129.60; 127.66; 126.56; 122.50; 116.91; 96.81; 63.55; 42.40; 33.35; 30.80; 26.43; 20.4; 15.02. Anal. Calcd for $C_{40}H_{38}N_2O_8$: C, 71.20; H, 5.68; N, 4.15; O, 18.97. Found: C, 71.25; H, 5.77; N, 4.11.

G-Quadruplex DNA Alkylation and Folding Induction. For reaction with DNA, compounds were dissolved and diluted in DMSO, in order to obtain a final 2.5% DMSO. DNA sequences (Biosense) were 4GGG, 5′-AGGGTTAGGGTTAGGGTTAGGG-3′; ss-scrambled-4GGG, 5′-GGATGTGAGTGTGAGTGTGAG-3′ and its complementary strand 5′-CTCACACTCACACTCACATCC-3′ to form ds-scrambled-4GGG; 2GGG, 5′-TACAGATAGT-TAGGGTTAGGGTTA-3′. For DNA labeling reactions, 100 pmol of DNA was incubated with 1 μL (10 μCi/μL) of [γ-^{32}P]ATP and 10 units of T4 polynucleotide kinase (Fermentas) in 50 mM TRIS-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, and 0.1 mM EDTA at 37 °C for 30 min. After incubation, DNA was purified through MicroSpin G-25 columns (GE Healthcare). For the alkylation experiment, 5−10 pmol of ^{32}P-labeled DNA was incubated with increasing drug amounts in phosphate buffer pH 7.4, 10 mM LiOH, and 50 mM KCl. Samples were incubated at the indicated temperature and time and reactions were stopped by precipitation. For competition experiments, annealed-labeled 4GGG (3.9 pmol) and drug (2 μM **5**) were mixed with cold competitor-DNA (4GGG, ss-scrambled-, and ds-scrambled-4GGG) at increasing concentrations, so that the molar ratios competitor/labeled 4GGG were 0.5, 1, 2, 5, and 10. After 24 h of incubation at 40 °C, samples were precipitated. Alkylation products were then resolved in 20% polyacrylamide 7 M urea sequencing gels. The NDI's ability to induce the G-quadruplex structure was tested by gel shift experiments. For G-4 folding induction, ^{32}P-labeled 2GGG and 5 μM cold 2GGG were annealed in phosphate buffer pH 7.4, containing 10 mM LiOH with or without 50 mM KCl. The annealed 2GGG was mixed with 25 μM of drug for increasing times at the indicated temperatures. Samples were loaded onto 16% polyacrylamide gel containing 20 mM KCl.

Exonuclease I Digestion. For exonuclease I reaction, the alkylation product was resolved in 20% polyacrylamide 7 M urea gel and purified by cutting and crushing the band of interest and eluting the alkylated oligo in TE buffer for 2 h. The purified alkyalted oligo was then precipitated and resuspended in exonuclease I reaction buffer. Exonuclease I was added at 10, 20, and 40 units and the reaction was incubated for 30 min at 37 or 50 °C. The reaction was stopped by DNA precipitation and samples were loaded onto 20% polyacrylamide 7 M urea gel.

Circular Dichroism Measurements. ligonucleotide 4GGG circular dichroism spectra from 230 to 350 nm were recorded using 10 mm path length cells on a Jasco J 810 spectropolarimeter equipped with a NESLAB temperature controller. Before data acquisition, a 4 μM 4GGG solution in Li_3PO_4 10 mM, KCl 50 mM, pH 7.4, was prepared, heated at 95 °C for 5 min, and left to cool at room temperature overnight. This solution was divided into four fractions: two were incubated for 24 h at 25 and 40 °C, respectively; **5** was added to a final concentration of 26 μM to the remaining fractions and incubated for 24 h at 25 and 40 °C, respectively. The reported spectrum of each sample represents the average of 3 scans recorded with 1-nm step resolution at 25 °C. Thermal denaturation experiments were performed recording the CD signal at 265 nm while increasing the temperature at 0.8 °C/min and stirring the DNA solution to allow equilibration.

Cytotoxicity Assay. Human embryonic kidney 293T cells (293T) were purchased from ATCC (ATCC number CRL-11268). 293T cells were grown as monolayers in Dulbecco's Modified Eagle Medium (Invitrogen, Italy) with 10% fetal bovine serum (FBS) supplemented with penicillin (100 U/mL) and

streptomycin (100 μg/mL) in a humidified atmosphere with 5% CO_2 at 37 °C.

Cytotoxic effects on cell growth were determined by MTT assay. NDI compounds were dissolved and diluted into working concentrations with DMSO. Cells (1.75×10^4 cells/well) were plated onto 96-microwell plates to a final volume of 100 μL and allowed an overnight period for attachment. At day 1, 1 μL of each dilution of tested compounds was added per well in order to get a 1% final concentration of drug solvent per well; at day 2, medium was removed, cells were washed with PBS, and fresh medium was added. Control cells (without any compound but with 1% drug solvent) were treated in the exact same conditions. Cell survival was evaluated by MTT assay: 10 μL of a freshly dissolved solution of MTT (5 mg/mL in PBS) was added to each well, and after 4 h of incubation, 100 μL of solubilization solution (10% SDS, 0,01 M HCl) was added. After overnight incubation at 37 °C, absorbance was read at 540 nm. Data were expressed as mean values of three individual experiments conducted in triplicate. The percentage of cell survival was calculated as follows: cell survival $= (A_{well} - A_{blank})/(A_{control} - A_{blank}) \times 100$, where blank denotes medium without cells.

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Supporting Information Available: Experimental and characterization details, ^{1}H and ^{13}C NMR spectra for the modified NDIs **4**−**6**, their related amines **4a**−**6a** and precursors, together with the NDI-QM alkylated adducts **7**−**20**. This material is available free of charge via the Internet at http://pubs.acs.org.

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