CHEMISTRY
A EUROPEAN JOURNAL

ChemPubSoc
Europe

DOI: 10.1002/chem.201203097

Targeting Loop Adenines in G-Quadruplex by a Selective Oxirane

Filippo Doria,[a] Matteo Nadai,[b] Marco Folini,[c] Matteo Scalabrin,[d] Luca Germani,[a] Giovanna Sattin,[b] Mariella Mella,[a] Manlio Palumbo,[e] Nadia Zaffaroni,[c] Daniele Fabris,[d] Mauro Freccero,*[a] and Sara N. Richter*[b]

G-quadruplexes (G4s) are DNA or RNA four-stranded supramolecular architectures that can form in G-rich regions. G4s have been found in biologically significant regions of the genome, such as telomeres, gene promoters, and in sequences associated with human disease.[1] Due to their critical role in key biological processes, G4s have been the object of intense study for their potential as therapeutic targets.[2] To date, a broad range of compounds have been identified as G4 ligands,[3] both in vitro and in vivo, with encouraging results in clinical trials.[2a] Among them, numerous tri- and tetra-substituted naphthalene diimides (NDIs) have shown high affinity for telomeric G4s and good antiproliferative activity.[4] In this context, we recently began the development of hybrid ligand-alkylating NDIs that possess a binding core tethered to an electrophile precursor, such as a quinone methide (QM, Scheme 1),[5] which can interact covalently with G4 structures.[4d–f] Covalent G4 targeting was also explored by using Pt^{II}–terpyridine complexes.[6] The strategy highlighted in Scheme 1 affords the possibility of triggering the alkylating activity under well-defined environmental conditions (e.g., light or mild digestion at 40 °C), which would help minimize typical off-target reactivity before the site of attack is reached.[7] Unfortunately, the QMs tested to date have yielded rather reversible DNA adducts, which eluded structural characterization.[4e,f] In the attempt to overcome such a drawback we have developed NDIs tethering the reversible ligands **1**–**4** (Scheme 1), to both a QM precursor (**5** and **6**) and an intrinsically reactive oxirane (**7**). The reversible binding of **1**–**4** and the alkylating properties of **5**–**7** were evaluated against a model G4 structure corresponding to the human telomere. The superior reactivity and selectivity of the alkylating oxirane **7** were unambiguously and unprecedentedly assessed by different mass spectrometric approaches.

The NDIs **1**–**6** were synthesized by a nucleophilic aromatic substitution (S_NAr), on the dibromo-NDI **8**,[8] in the presence of 4-(2-aminoethyl) phenol and 4-ethenyl benzenemethanamine for **1**–**6** and **7**, respectively (Supporting Information, Scheme S1). The desired side chains and the alkylating moieties were introduced only subsequently to the S_NAr, in order to preserve their structural integrity. In this way, the multistep synthesis for **1**–**4** involved an S_NAr and a Mannich reaction, followed by an exhaustive methylation of the amines **3** and **4**, yielding the quaternary ammonium salts (**5**, **6**). The oxirane **7** was obtained as a racemic mixture by S_NAr with the use of 4-vinylbenzylamine and subsequent epoxidation with dimethyldioxirane (DMD) or *meta*-chloroperbenzoic acid (MCPBA, yield > 90 %).

The activity of reversible ligands **3** and **4** was initially assayed by mixing them with a labeled oligonucleotide that reproduced the human telomeric DNA sequence (F21T; Supporting Information, Figure S1) and by using FRET to measure the melting of the structure.[9] Addition of 1.0 μM NDIs to a 0.25 μM solution of F21T in the presence of K^+ induced melting temperature increases (ΔT_m) of 10(±1.0) °C and 11(±0.5) °C for **3** and **4**, respectively. In contrast, no T_m variations were observed upon incubation with labeled double-stranded DNA. Possible structural perturbations were also monitored by circular dichroism (CD).[10] In this case, minor spectral variations were detected when the NDIs were added to a fully-folded hTel (unlabelled human telomeric sequence), which displayed the typical G4 fingerprint with a maximum at 290 nm. When the compounds were added before folding, an additional maximum was noted at 260 nm (Supporting Information, Figure S2). This behavior indicates that the NDIs may partially direct the folding towards a parallel-like conformation, as described for other NDIs.[4e,f] The continuous variation method (Job plot)[11] was employed to evaluate the 1:1 binding stoichiometry (Supporting Information, Figure S2).

[a] Dr. F. Doria,[+] Dr. L. Germani, Prof. M. Mella, Prof. M. Freccero
Dipartimento di Chimica, Università di Pavia
V.le Taramelli 10, 27100 Pavia (Italy)
E-mail: mauro.freccero@unipv.it

[b] Dr. M. Nadai,[+] Dr. G. Sattin, Dr. S. N. Richter
Dipartimento di Medicina Molecolare
Università di Padova, via Gabelli 63, 35121 Padua (Italy)
E-mail: sara.richter@unipd.it

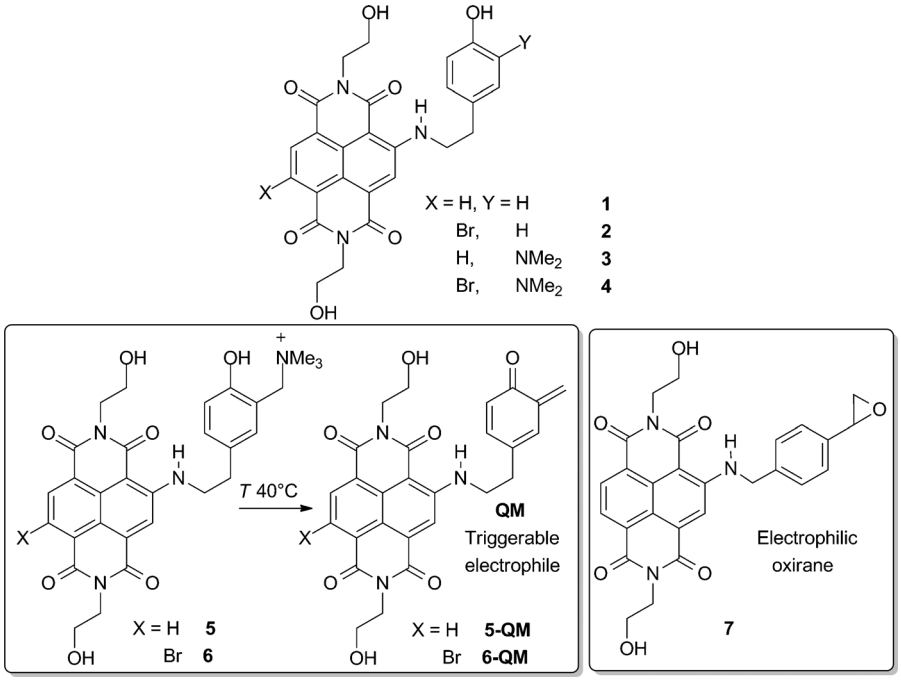
[c] Dr. M. Folini, Dr. N. Zaffaroni
Dipartimento di Oncologia Sperimentale e Medicina Molecolare
Fondazione IRCCS Istituto Nazionale dei Tumori
Via G. Amadeo 42, 20133 Milano (Italy)

[d] Dr. M. Scalabrin, Dr. D. Fabris
The RNA Institute, University at Albany
1400 Washington Ave., Albany, NY 12222, USA

[e] Prof. M. Palumbo
Department of Pharmaceutical and Pharmacological Sciences
Università di Padova, Via Marzolo 5, 35131 Padova (Italy)

[+] These authors contributed equally to this work.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201203097.



Scheme 1. NDIs as reversible (**1**–**4**) and alkylating G4 binders (**5**–**7**).

The alkylating properties of **5** and **6** were assayed by incubating them with selected substrates at 40 °C and by monitoring adduct formation by denaturing polyacrylamide gel electrophoresis (PAGE). Reactivity was tested against the folded hTel, single-stranded (ss) and double-stranded (ds) scrambled hTel sequences (Supporting Information, Figure S1). Adduct formation was observed at a molar ratio of 0.8:1 NDI/hTel, and yields increased to approximately 10% adduct at a ratio of 12:1 (Figure 1A and B). In contrast, the same amount of ss substrate failed to produce detectable adducts even when treated with up to 50:1 NDI/ss (Figure 1B). Similarly, the ds substrate produced only a modest, approximately 2%, adduct when the analogue concentrations were increased to 200:1 NDI/ds (Figure 1B, and Supporting Information, Figure S3). Under the same conditions, the oxirane **7** displayed greater reactivity than the corresponding QM activatable counterparts (Figure 1C). In this case, up to approximately 16% adduct yield was obtained at 12:1 NDI/hTel (Figure 1D). Conversely, alkylation of ss and ds substrates was modest (<2%, when treated with up to 200:1 NDI/substrate). The negligible reactivity manifested by substrates that do not fold into G4 suggests a mechanism involving specific substrate–ligand recognition before the actual alkylation can take place. Substrates that are unable to sustain NDI binding appear incapable of undergoing significant alkylation, even though they still contain putative reactive sites of A and G.

These data indicate a selective alkylation of the G4 structure over unstructured or double helix DNA. The possible reversible character of the alkylated G4 product was explored as a function of temperature and salt concentration. The adduct was stable after 10 min incubation at 95 °C and in up to 1.0 M KCl solution (Supporting Information, Figure S4). By comparison, these conditions were found sufficient to partially revert adducts formed by the activatable QM compound **6** (Supporting Information, Figure S5). The higher efficiency in the modification of hTel by **7** in comparison to **6** (Figure 1A vs. Figure 1C) and the increased stability of the resulting **7**–hTel (Supporting Information, Figure S4) in comparison to **6**–hTel (Supporting Information, Figure S5), prompted us to further study the nature of the former. Therefore, the structure of the **7**–hTel adduct was investigated by electrospray ionization mass spectrometry (ESI-MS), which was performed on both unreacted and alkylated species after isolation by PAGE. The observed mass of 7464.33 Da matched very closely the mass of 7464.34 Da calculated for a 1:1 adduct by adding the masses of the initial G4 construct and **7** (Supporting Information, Figure S6). These data suggest that the putative adduct was produced by the opening of the oxirane induced by hTel. When submitted to tandem mass spectrometry (MS/MS), the alkylated product provided typical fragment ions that matched the sequence of hTel. As typically observed for covalent adducts of nucleic acids,[13] the fragments included a characteristic mass shift corresponding to analogue **7** (i.e., 501 Da incremental mass), which enabled us to locate its putative position at A1, A7, A13, and A19 (Figure 2). The detection of a fragment corresponding to the adduct **7** bound to an A nucleobase (i.e., *m*/*z* 637.2, Supporting Information, Figure S7A) was consistent with the mechanism of DNA fragmentation, which is prompted by initial base loss.[13] A similar alkylation selectivity has been reported for Pt^{II}–terpyridines, which coordinate exclusively the adenine nucleobases present in the G4 loops.[6] Considering the well-known styrene oxide reactivity towards all four deoxyribonucleosides dG > dC > dA ≫ dT[14] it was surprising to observe that A exhibited greater susceptibility than G. In contrast, the alkylated adduct of **7** with G nucleobase was detected when the substrate considered was the ss construct with scrambled sequence. Indeed, a fragment corresponding to alkylated G was recognizable in the MS/MS spectrum of the ss-DNA/oxirane adduct (Supporting Information, Figure S7B). At the same time, no product of A alkylation could be detected; this suggests that adduct formation was profoundly affected by the structural context.

In conclusion, we have developed two types of G4 alkylating agents. Both alkylation efficiency and selectivity were

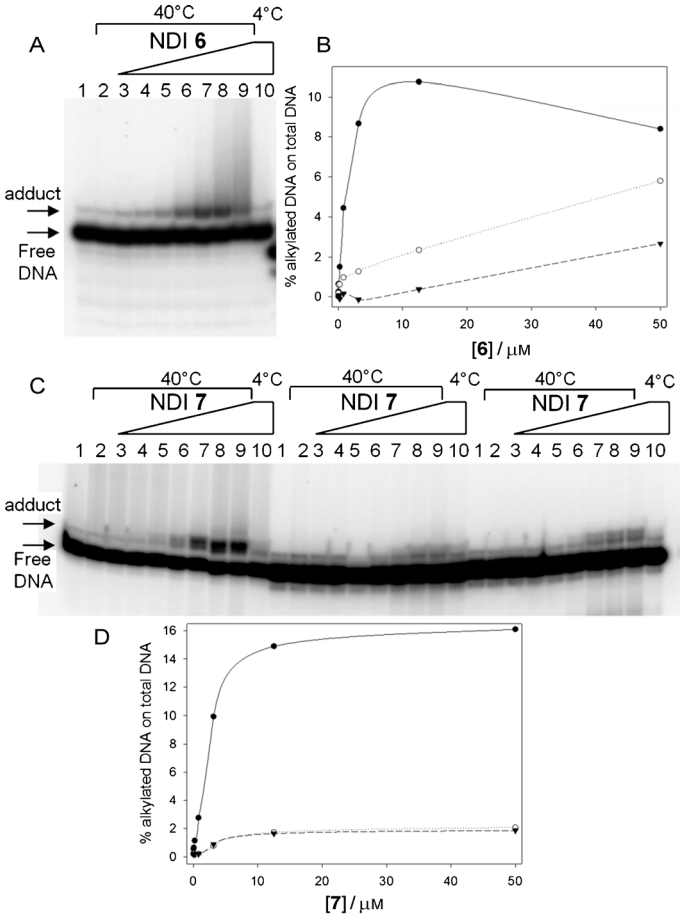


Figure 1. Alkylation of **6** and **7** analyzed by PAGE. A) ^{32}P-labeled hTel alone (lane 1) and digested with **6** at 40 °C for 24 h (lanes 2–9) or at 4 °C for 24 h (lane 10). B) Quantification of adduct bands obtained by treating hTel (●), ss- (○) and ds-scrambled hTel (▼) with compound **6**. C) Compound **7** with ^{32}P-labeled hTel, ss- and ds-scrambled hTel. D) Quantification of adduct bands obtained by treating hTel (●), ss- (○) and ds-scrambled hTel (▼) with compound **7**.

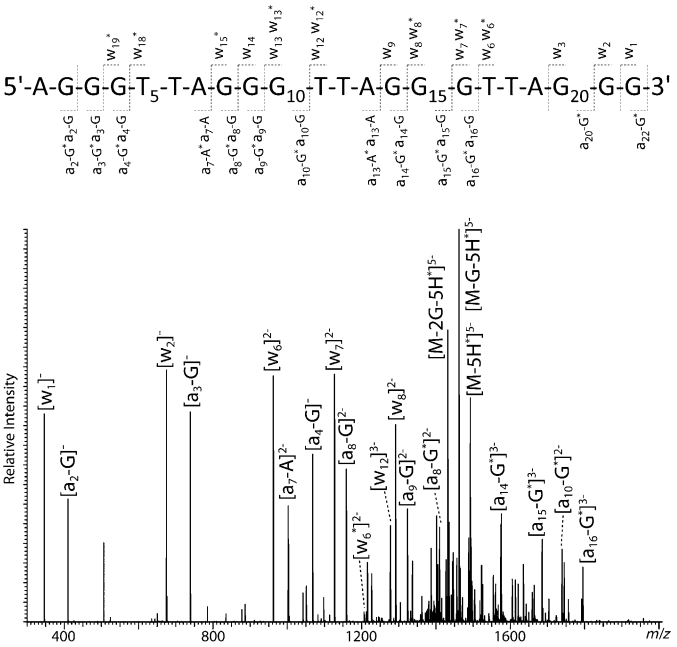


Figure 2. MS/MS spectrum of **7**–hTel adduct obtained in negative ion mode. Characteristic ion series are labeled according to standard nomenclature;[12] $[M-5H]^{5-}$ indicates the precursor ion at *m/z* 1492.46. Ions marked with an asterisk included a 501 Da mass shift from the corresponding unmodified fragment, in agreement with the presence of one unit of **7**. All fragments are summarized on the hTel sequence to enable a direct comparison of ion series with (marked with an asterisk) and without (unmarked) adduct.

enhanced in the oxirane derivative. Although QM adducts proved to be insufficiently stable for isolation and characterization, the stability of oxirane adducts enabled MS analysis. The results showed a highly selective alkylation of A versus G in hTel, which is consistent with a lower G accessibility within the G4 structure. The presence of A nucleobases in the loop regions of most oncogene promoters that form G4s[15] suggests that NDI–oxirane conjugates may covalently target different cancer-related G4 structures based on its affinity for G-quartets and selective alkylation of adenines.

Acknowledgements

Financial support from MIUR, Rome (FIRB-IdeasRBID082ATK, and PRIN 2009MFRKZ8), University of Pavia, University of Padua, and the National Institutes of Health (GM064328-12 to D.F.) is gratefully acknowledged.

Keywords: alkylation · DNA damage · G-quadruplexes · oxirane · quinone methides

[1] a) J. L. Huppert, S. Balasubramanian, *Nucleic Acids Res.* **2005**, *33*, 2908–2916; b) J. L. Huppert, S. Balasubramanian, *Nucleic Acids Res.* **2006**, *35*, 406–413.
[2] a) S. Balasubramanian, L. H. Hurley, S. Neidle, *Nat. Rev. Drug. Discov.* **2011**, *10*, 261–275; b) K. Paeschke, T. Simonsson, J. Postberg, D. Rhodes, H. J. Lipps, *Nat. Struct. Mol. Biol.* **2005**, *12*, 847–854.
[3] a) A. De Cian, P. Grellier, E. Mouray, D. Depoix, H. Bertrand, D. Monchaud, M. P. Teulade-Fichou, J. L. Mergny, P. Alberti, *ChemBioChem* **2008**, *9*, 2730–2739; b) M. Petenzi, D. Verga, E. Largy, F. Hamon, F. Doria, M.-P. Teulade-Fichou, A. Guédin, J.-L. Mergny, M. Mella, M. Freccero, *Chem. Eur. J.* **2012**, *18*, 14487–14496.
[4] a) G. W. Collie, R. Promontorio, S. M. Hampel, M. Micco, S. Neidle, G. N. Parkinson, *J. Am. Chem. Soc.* **2012**, *134*, 2723–2731; b) M. Gunaratnam, M. de La Fuente, S. M. Hampel, A. K. Todd, A. P. Reszka, A. Schätzlein, S. Neidle, *Bioorg. Med. Chem.* **2011**, *19*, 7151–7157; c) S. M. Hampel, A. Sidibe, M. Gunaratnam, J. F. Riou, S. Neidle, *Bioorg. Med. Chem. Lett.* **2010**, *20*, 6459–6463; d) M. Di Antonio, F. Doria, S. N. Richter, C. Bertipaglia, M. Mella, C. Sissi, M. Palumbo, M. Freccero, *J. Am. Chem. Soc.* **2009**, *131*, 13132–13141; e) M. Nadai, F. Doria, M. Di Antonio, G. Sattin, L. Germani, C. Percivalle, M. Palumbo, S. N. Richter, M. Freccero, *Biochimie* **2011**, *93*, 1328–1340; f) F. Doria, M. Nadai, M. Folini, M. Di Antonio, L. Germani, C. Percivalle, C. Sissi, N. Zaffaroni, S. Alcaro, A. Artese, S. N. Richter, M. Freccero, *Org. Biomol. Chem.* **2012**, *10*, 2798–2806.
[5] *Quinone Methides* (Ed.: S. E. Rokita), Wiley, New York, **2009**.

[6] H. Bertrand, S. Bombard, D. Monchaud, E. Talbot, A. Guedin, J. L. Mergny, R. Grunert, P. J. Bednarski, M. P. Teulade-Fichou, *Org. Biomol. Chem.* **2009**, *7*, 2864–2871.
[7] a) F. Doria, C. Percivalle, M. Freccero, *J. Org. Chem.* **2012**, *77*, 3615–3619; b) D. Verga, M. Nadai, F. Doria, C. Percivalle, M. Di Antonio, M. Palumbo, S. N. Richter, M. Freccero, *J. Am. Chem. Soc.* **2010**, *132*, 14625–14637.
[8] F. Doria, M. di Antonio, M. Benotti, D. Verga, M. Freccero, *J. Org. Chem.* **2009**, *74*, 8616–8625.
[9] A. De Cian, L. Guittat, M. Kaiser, B. Sacca, S. Amrane, A. Bourdoncle, P. Alberti, M. P. Teulade-Fichou, L. Lacroix, J. L. Mergny, *Methods* **2007**, *42*, 183–195.
[10] A. I. Karsisiotis, N. M. Hessari, E. Novellino, G. P. Spada, A. Randazzo, M. Webba da Silva, *Angew. Chem.* **2011**, *123*, 10833–10836; *Angew. Chem. Int. Ed.* **2011**, *50*, 10645–10648.
[11] C. Y. Huang, *Methods Enzymol.* **1982**, *87*, 509–525.
[12] S. A. McLuckey, S. Habibi-Goudarzi, *J. Am. Chem. Soc.* **1993**, *115*, 12085–12095.
[13] a) E. Nordhoff, F. Kirpekar, P. Roepstorff, *Mass Spectrom. Rev.* **1996**, *15*, 67–138; b) K. A. Kellersberger, E. Yu, G. H. Kruppa, M. M. Young, D. Fabris, *Anal. Chem.* **2004**, *76*, 2438–2445.
[14] K. Savela, A. Hesso, K. Hemminki, *Chem.-Biol. Interact.* **1986**, *60*, 235–246.
[15] T. A. Brooks, S. Kendrick, L. Hurley, *FEBS J.* **2010**, *277*, 3459–3469.

Received: August 31, 2012
Published online: December 4, 2012

