CHEMISTRY
A European Journal

A Journal of
ChemPubSoc Europe

Accepted Article

Title: Synthesis, binding properties and differences on cell uptake of G-quadruplex ligands based on carbohydrate naphthalene diimide conjugates

Authors: Matilde Arevalo, Filippo Doria, Efres Belmonte-Reche, Aurore De Rache, Jenny Campos-Salinas, Ricardo Lucas, Eva Falomir, Miguel Carda, Jose María Pérez-Victoria, Jean-Louis Mergny, Mauro Freccero, and Juan Carlos Morales

To be cited as: *Chem. Eur. J.* 10.1002/chem.201604886

Link to VoR: http://dx.doi.org/10.1002/chem.201604886

Supported by
ACES

WILEY-VCH

10.1002/chem.201604886

Synthesis, binding properties and differences on cell uptake of G-quadruplex ligands based on carbohydrate naphthalene diimide conjugates

Matilde Arévalo-Ruiz†,[a] Filippo Doria†,[b] Efres Belmonte-Reche,[a] Aurore De Rache, [c] Jenny Campos-Salinas,[a] Ricardo Lucas,[a] Eva Falomir,[d] Miguel Carda,[d] José María Pérez-Victoria,[a] Jean-Louis Mergny, [c] Mauro Freccero * [b] and Juan Carlos Morales *[a]

Affiliations:

[a] Department of Biochemistry and Molecular Pharmacology, Instituto de Parasitología y Biomedicina, CSIC, Parque Tecnológico Ciencias de la Salud, Avda. del Conocimiento, s/n, 18016 Armilla, Granada, Spain

[b] Department of Chemistry, University of Pavia , V.le Taramelli 10, 27100 Pavia, Italy.

[c] Institut Européen de Chimie Biologie (IECB), ARNA laboratory, Université de Bordeaux, Inserm U1212, CNRS UMR5320, 2 rue Robert Escarpit, Pessac, France.

[d] Department of Inorganic and Organic Chemistry, University Jaume I, 12071 Castellón, Spain

Note: † These authors contributed equally to this work.

E-mail corresponding authors:

mauro.freccero@unipv.it

jcmorales@ipb.csic.es

10.1002/chem.201604886

Abstract

G-quadruplexes (G4) are currently being explored as therapeutic targets in cancer and other pathologies. We have synthesized six carbohydrate naphthalene diimide conjugates (carb-NDIs) as G4 ligands to investigate their potential selectivity on G4 binding and on cell penetration. Carb-NDIs have shown certain selectivity for G4 structures against DNA duplexes but the different sugar moieties did not induce a preference for a specific G4 topology. Interestingly, when monosaccharides were attached through a short ethylene linker to the NDI scaffold their cellular uptake was 2-3 fold more efficient than when the sugar was directly attached through its anomeric position. Moreover, a correlation between the more efficient cell uptake of these carb-NDIs and their higher toxicity in cancerous cell lines has been observed. Carb-NDIs seem to be mainly translocated into the cancer cells through glucose transporters where GLUT4 plays a major role.

10.1002/chem.201604886

Introduction

G-quadruplexes (G4) are non-canonical higher-order nucleic acid structures that form on repeats of short guanine(G)-tracts. Their recurrent structural motif is made by planar arrangement of four strands of guanine bases stacked upon one another and a monovalent cation among them.[1] Currently, quadruplexes are viewed as emerging therapeutic targets in cancer[2] as well as in neurological disorders[3] and viral infections.[4] The central hypothesis on the field is that stabilizing a promoter G4 through small molecule binding brings inhibition of the transcriptional machinery and down-regulation of expression of the targeted gene.[5] However, several important challenges remain if G4 ligands are to be therapeutically-useful compounds, including: selectivity against the much more abundant double-stranded DNA, selectivity between different G4 structures and/or topologies and selectivity towards cellular targets.

We previously studied carbohydrate-DNA interactions in double helices and in G4 using carbohydrate oligonucleotide conjugates.[6] We found that carbohydrate-thrombin binding aptamer conjugates showed stacking interactions between the sugar and a DNA guanine tetrad together with hydrogen bonding and hydrophobic contacts.[7] Di Antonio *et al.*[8] explored this possibility by conjugation of neutral carbohydrates to G4 ligands resulting in the identification of stronger and more selective DNA G4 binders. Similarly, isoflavones such as glycosidic daidzin were reported as G4 ligands capable of inducing a structural change of the polymorphic human telomeric G4 (hTel) into its antiparallel conformation.[9] Recently, N-phenanthroline glycosylamine copper(II) complexes have been reported to bind hTel with an important contribution of groove and/or loop contacts.[10] Aminosugars such as neomycin have also been attached to different aromatic rings to prepare G4 ligands.[11]

In the last decade, a large number of studies have exploited the naphthalene diimide moiety (NDI) as a G4-binding motif, taking advantage of its synthetic accessibility,

optoelectronic,[12] G4 binding[13] and sensing properties.[14] Tri- and tetra-substituted NDIs have been broadly explored NDIs due to their potency and duplex *vs* G4 selectivity, and have proven to be effective cores for multimodal reactive[15] and photoreactive NDI-conjugates,[16] and even targeting human medullary thyroid cancer.[17] Tetra-substituted NDIs have shown bimodal fluorescence and photocytotoxicity,[18] high affinity for human telomeric G4 DNA,[19] antiproliferative potency in pancreatic cancer cells[13c, 20] and inhibition of telomerase activity.[21]

In this work we have designed and synthesized a family of carbohydrate-G4 ligand conjugates based on a substituted naphthalene diimide scaffold (carb-NDIs, Figure 1). By sugar conjugation to di-charged substituted NDIs, we reduced the overall positive charge in comparison to previously investigated tri-charged and tetra-charged substituted NDIs, preserving their solubility and the fluorescence emission of the NDI core, which is centred at 595 nm and 661 nm, respectively.[12a] We selected to attach glucose using different presentations (β-glc, β-glcC2 and β-malt), and three other monosaccharides (β-glcNAc, β-6deoxyglc and α-manC2) to di-charged substituted NDIs in order to explore their potential different binding to G4 targets and their different capacity to be transported through GLUT.

From this conjugation we expected to increase compound solubility and bioavailability, improve G4-binding through sugar-mediated interactions to the phosphates and G4 DNA grooves and finally, promote selective entrance into tumor cells through overexpressed glucose transporters (GLUT). This approach has been explored in other cytotoxic drugs by taking advantage of the Warburg effect and the energy requirements for rapid growth displayed by aggressive cancerous cells.[22] Examples include glucose-paclitaxel,[23] 2-amino-2-deoxyglucose adriamycin,[24] glucose-lactate dehydrogenase inhibitors,[25] glucose-platinum conjugates[26] and

10.1002/chem.201604886

glufosfamide[27] which has recently advanced to metastatic pancreatic cancer Phase III clinical trials.

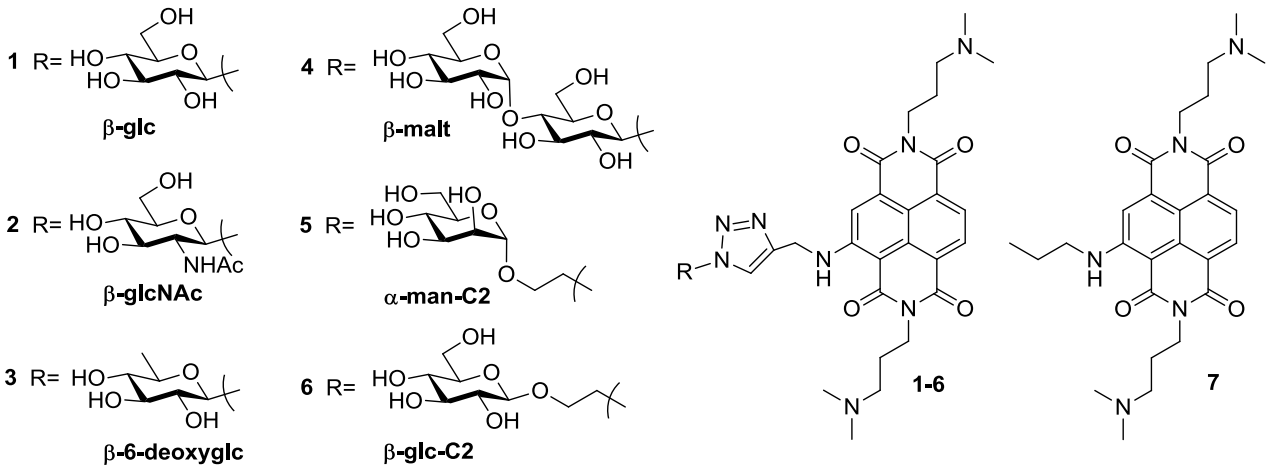


Figure 1. Carb-NDI conjugates **1**-**6** prepared and investigated in this work together with the aglycone NDI **7**.

Results and discussion.

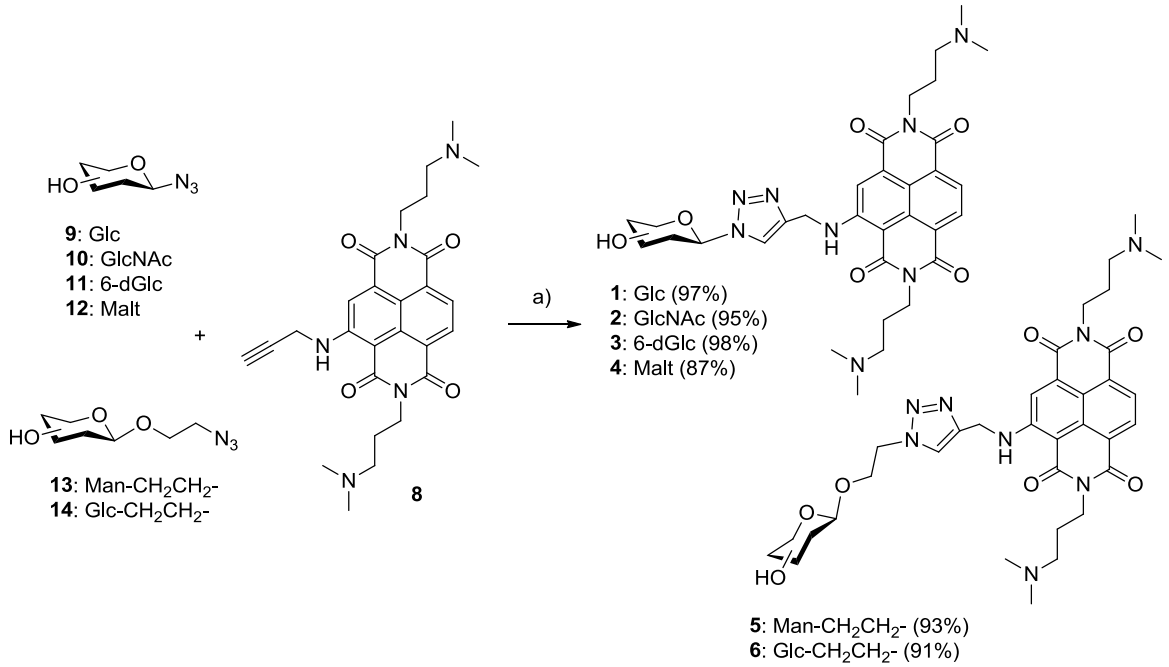
Design and synthesis of naphthalene diimide carbohydrate conjugates.

The synthesis of the new carbohydrate-triazole-NDI conjugates **1**-**6** were carried out by copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) click chemistry. A series of azido glycosides building blocks with focused structural diversity were initially synthesized and then clicked onto 2-N-propargyl naphthalene diimide **8** (Scheme 1). The preparation of the new propargyl-NDI **8** relies on the imidation reaction of the commercially available 2,6-dibromo-1,4,5,8-naphthalenetetracarboxylic dianhydride by N',N'-dimethyl-1,3-propanediamine, giving quantitatively the N,N′-Bis((dimethylamino)propylamino)-2,6-di-bromo-1,4,5,8-

10.1002/chem.201604886

naphthalenetetracarboxylic bisimide (Scheme S1). The subsequent nucleophilic aromatic substitution (S_NAr) in the presence of an excess (2.5 eq.) of propargylamine (CH_3CN as solvent, 75 °C, 4.5 h) afforded NDI **8** as the only product with quantitative conversion. Azido glycosides and 2-azidoethyl glycosides were synthesized as described previously.[28] Briefly, acetobromo sugars reacted with sodium azide and the following deprotection under basic conditions yielded the corresponding azido glycosides. The 2-azidoethyl glycosides were obtained by a glycosylation with 2-bromoethanol, followed by reaction with sodium azide and treatment with sodium methoxide.[29] The carb-NDIs **1-6** were synthesized by Cu(I) catalyzed Huisgen 1,3-dipolar cycloaddition between propargyl-NDI **8** and azide building blocks **9-14** (Scheme 1). The "click" reaction was performed under typical conditions, using $CuSO_4 \cdot 5H_2O$, sodium ascorbate in a t-BuOH/H_2O mixture (1:1) and stirring for 2 hrs at r.t., under nitrogen atmosphere. The reactions proceeded smoothly and final carb-NDIs derivatives were purified by preparative HPLC. Aglycone NDI **7** was prepared as reference ligand, in order to compare the biophysical and biological properties of the new carb-NDI conjugates with a sugar-free NDI analogue (see Scheme S2, Sup. Inf.).

10.1002/chem.201604886



Scheme 1. Synthesis of the new carb-NDI conjugates **1**-**6**, by CuAAC reaction; a) Na ascorbate, $CuSO_4$, ^tBuOH / H_2O (1:1), r.t.

G4 Binding studies

FRET-melting assays. The binding of ligands **1**-**7** to G4 structures was first investigated using FRET-based melting assays on the F21T human DNA telomeric oligonucleotide [30] in the presence of K^+ (Figure 2a). All ligands induced an increase of the melting temperature ($T_{1/2}$) of the G4 structure. This stabilization is expressed as the difference in melting temperature with and without ligand ($\Delta T_{1/2}$). The NDI-sugar conjugates had an important stabilization effect on the F21T G4, with $\Delta T_{1/2}$ values close to 20 °C at 2 µM compound concentration, which was almost identical to the stabilization induced by the control ligand **7** which has no carbohydrate in its structure. Therefore, with the exception of compound **6**, the sugar moiety did not introduce any detrimental effect on G4 stabilization. Carb-NDI $\Delta T_{1/2}$ values were slightly lower than $\Delta T_{1/2}$ values reported previously for tri-substituted NDIs with a conjugated Mannich base ($\Delta T_{1/2}$ =22°C)[31] or charged tertiary amines under physiological conditions ($\Delta T_{1/2}$ =26°C).

10.1002/chem.201604886

Nevertheless, a direct comparison of our $\Delta T_{1/2}$ values and literature results can be misleading, since different ligand and K^+ concentration have been used.

In the presence of 3 µM of duplex competitor (15 equivalents), the induced stabilization dropped by approximatively 6 °C for all ligands. With 10 µM of competitor (50 equivalents), the induced stabilization was even lower but remained significant. These results demonstrate some selectivity of the ligands for G4 against DNA duplexes. This selectivity was confirmed using a variety of labeled oligonucleotides (see below).

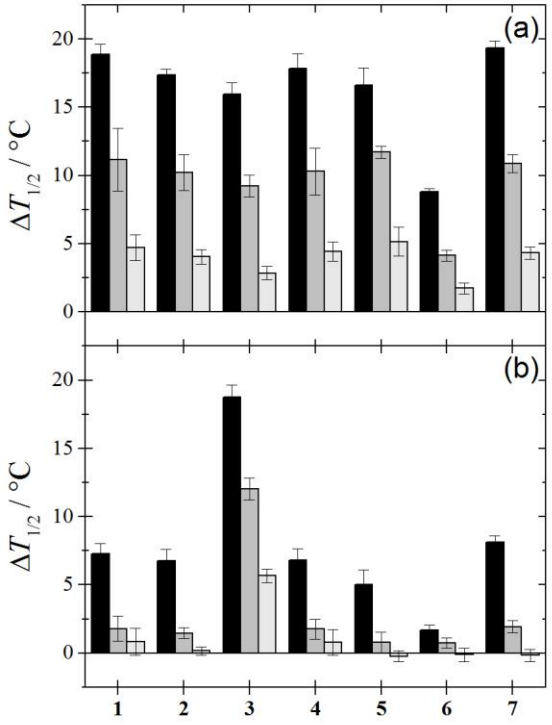


Figure 2. FRET-melting competition assay. Thermal stabilization induced by the tested compounds (2 µM) on the human telomeric quadruplex F21T (0.2 µM) in 10 mM lithium cacodylate pH 7.2 containing (a) 10 mM KCl and 90 mM LiCl or (b) 100 mM NaCl. The duplex competitor (ds26) strand concentrations are 0 (black), 3 (gray) and 10 µM (light gray).

10.1002/chem.201604886

The human telomeric sequence is known to adopt different conformations in Na^+-(antiparallel)[32] and K^+-media (parallel,[33] anti-parallel,[34] hybrid or [3+1][35]). Hence, G4-stabilization values were also calculated in presence of Na^+ as an initial indication of ligand G4-topology selectivity (Figure 2b). All ligands showed significantly lower Na^+-$\Delta T_{1/2}$ values than those observed in K^+ suggesting that the compound-induced stabilization depends on the G4 topology.

In order to provide further insight into the structural dependencies of the ligand binding, FRET melting selectivity profiles (Figure 3) were built from the $\Delta T_{1/2}$ values obtained with a set of sequences including a duplex and five G4s (Table 1 and Figure S1).[36] The choice of the oligonucleotide sequences aimed at representing the structural and topological diversity of G4. Duplex sequence (FdxT) stabilization was negligible for all ligands in comparison to that of the studied G4 which is in agreement with the results obtained in the competitive assay. No clear selectivity was observed for a given target although compounds **1**-**6** showed higher stabilization of the polymorphic human telomere G4 structures.

10.1002/chem.201604886

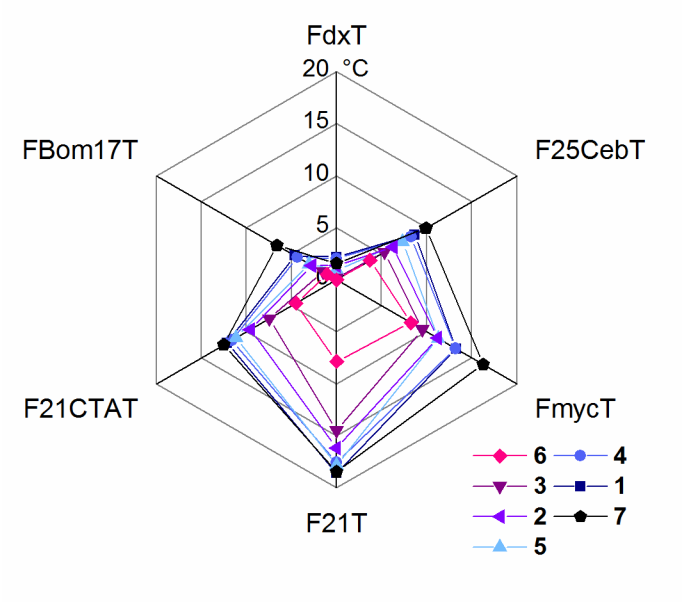


Figure 3. Selectivity profile. FRET-melting stabilization induced by the tested compounds (2 µM) on G-quadruplexes of various topologies in 10 mM lithium cacodylate pH 7.2 containing 10 mM KCl and 90 mM LiCl. Details on the sequences and structures of the G4 used are provided in Supporting Information (Table S1).

CD titrations. FRET melting experiments showed the highest thermal stabilisation was observed on the polymorphic human telomeric sequence F21T. In order to assess that the observed stabilisation was due to an interaction with the G4 (and not to the fluorescent dyes), we performed CD-titrations on the unlabelled 21g sequence (d-$(GGGTTA)_3GGG$) with ligands **1**, **4**, **6** and **7** (Figure 4). In all four cases, the interaction of the ligand with 21g was evidenced by an intensity increase of the 287 nm positive peak (typical of the [3+1] topology), without any change in the G4 folding. This is an interesting aspect differentiating the carb-NDIs from other NDIs such as the tri-substituted NDIs embedding amines, since the latter had a tendency to induce a partial refolding into a parallel topology.[37] Furthermore, no free-ligand CD-signature was

observed at this wavelength (Figure S2) confirming that the observed CD-changes reflect an interaction between the ligands and the G4 structure formed by the 21g sequence.

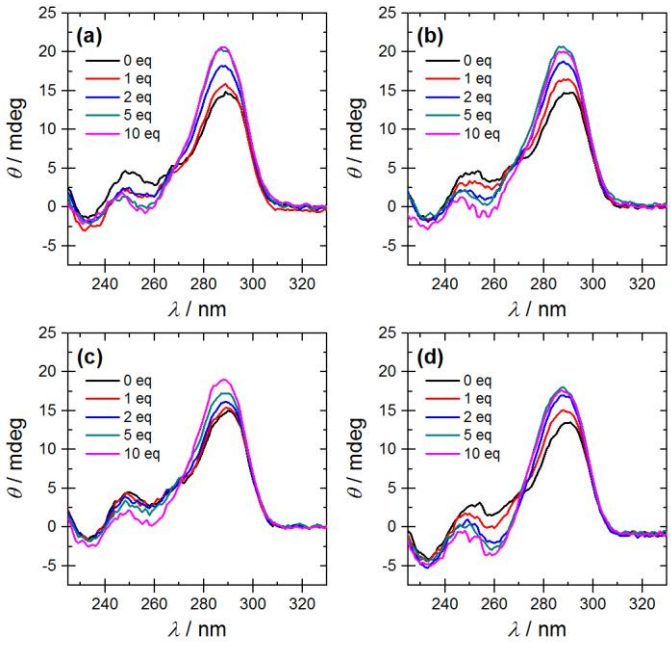


Figure 4. CD Titration of 21g with compounds β-glc-NDI **1** (a), β-malt-NDI **4** (b), β-glcC2-NDI **6** (c) and NDI-prop **7** (d). The oligonucleotide concentration was 4 μM.

Biological Studies.

Cell uptake studies.

We selected three structurally different NDI derivatives, β-glc-NDI **1** with a sugar directly attached to the azide unit, α-manC2-NDI **5** with a mannose linked through a spacer and aglycone-NDI **7** with no attached carbohydrate to study their entrance within two cancer cell lines (human colon adenocarcinoma HT-29 and breast adenocarcinoma MCF-7) and a non-cancerous cell line (human lung fibroblast MRC-5). We took advantage of the red fluorescent emission of the NDI derivatives **1**-**7** to monitor their cell entry. Using flow cytometry and

10.1002/chem.201604886

incubating these cell lines at different NDI-ligand concentrations (1 and 5 μM), different times (5 min, 1, 2 and 4 h) and two temperatures (4 and 37 ºC) we detected a quick entrance of sugar-free NDI **7** in all cell lines independent of the temperature and times assayed (similar uptake at 4 ºC after 5 minutes and at 37 ºC after 4 h) (Figure 5 and Figures S3-S6) suggesting uptake by passive diffusion or facilitated transport. In contrast, at 4 ºC β-glc-NDI **1** barely penetrated into the cells, whilst α-manC2-NDI **5** was incorporated slightly better, being more efficient in its cellular transport. When the temperature was increased to 37 ºC, carb-NDI ligands β-glc-NDI **1** and α-manC2-NDI **5** were incorporated much more efficiently than at 4 ºC indicating a temperature dependent mechanism of cell uptake operating in the process. Moreover, α-manC2-NDI **5** seems to enter the cells more efficiently than β-glc-NDI **1**.

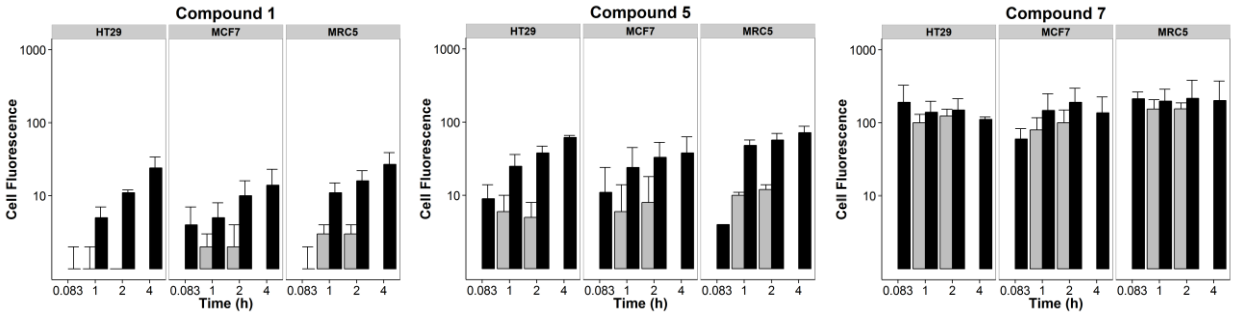


Figure 5. The mean fluorescence intensity of β-glc-NDI **1**, α-manC2-NDI **5** and aglycone-NDI **7** incubated at 5 μM concentration with various cancer and normal cell lines for 5 min, 1h, 2h and 4h measured by flow cytometry. Bars in grey, T= 4 ºC; bars in black, T= 37 ºC.

We carried out intracellular quantification of β-glc-NDI **1**, α-manC2-NDI **5**, β-glcC2-NDI **6** and aglycone-NDI **7** in order to confirm the apparent differences observed in cellular uptake among the three ligands. Fluorescence spectroscopy measurements were made directly

10.1002/chem.201604886

irradiating the red dye 485 nm and recording its emission at 535 nm after incubating the compounds (5 μM conc.) for 2h (Table 1). As expected from the flow cytometry studies, we observed higher intracellular concentrations of control aglycone-NDI **7** than in the case of carb-NDIs **1, 5** and **6** in all three cell lines examined. At the same time, cellular uptake of α-manC2-NDI **5** and β-glcC2-NDI **6** was considerably more efficient (2-3 fold) than for β-glc-NDI **1.** Compounds **5** and **6** both comprise a monosaccharide linked through an ethylene glycol spacer to the azide-NDI scaffold, whereas β-glc-NDI **1** exhibits the glucose directly linked to the azide-NDI structure, confirming the tendency observed by flow cytometry, on HT-29 and MRC-5 cells but not on MCF-7 cells (0.8-1.4 fold). Our results contrast somehow with recent studies by Patra et al.[26b] using glucose-platinum conjugates. Opposite to our findings, they concluded that increasing the length of the linker decreased cell uptake. However, the different nature of the core -and the different dimensions- may also influence the transport mechanism what could explain these divergent results.

	Cell line		
Compound	**HT-29**	**MCF-7**	**MRC-5**
β-glc-NDI **1**	453 ± 65 (30%)	526 ± 120 (28%)	236 ± 53 (13%)
α-manC2-NDI **5**	1093± 159 (73%)	439 ± 159 (23%)	545 ± 176 (30%)
β-glcC2-NDI **6**	988 ± 121 (66%)	762 ± 241 (41%)	778 ± 269 (43%)
NDI-prop **7**	1492 ± 508 (100%)	1858 ± 239 (100%)	1773 ± 98 (100%)

Table 1. Compound concentration (nM) per well and percentage of compound entering the cell relative to compound **7** (between brackets) of β-glc-NDI **1**, α-manC2-NDI **5**, β-glcC2-NDI **6** and aglycone-NDI **7** as measured by fluorescence spectroscopy. 225,000 cells were incubated with 5

10.1002/chem.201604886

μM of each NDI-Compound in 750 μL of medium for 2 h at 37 ºC. After centrifugation, supernatant removal and washing, the cell pellet was re-suspended in 750 μL of SDS 0.4 % and homogenized. Fluorescence values were normalized via protein quantification assay using Pierce BCA test (ThermoFisher Scientific) and concentration values were extrapolated from a fluorescence – NDI-concentration calibration curve.

Glucose transporters (GLUT) are likely to be the proteins associated to the cellular transport of the carb-NDIs ligands. So, we examined this possibility by quantifying the entrance of carb-NDI (**1**, **5** and **6**) and control aglycone-NDI **7** into cancerous HT-29 cells in the absence or presence of several GLUT inhibitors including: WZB117, a specific GLUT1 inhibitor;[38] ritonavir, a specific GLUT4 inhibitor[39] and flavonoids quercetin and genistein reported as dual GLUT1 and GLUT4 inhibitors.[40] We observed clear cell uptake inhibition for the three carb-NDIs **1**, **5** and **6** (35-64% inhibition) when quercetin was used as inhibitor (Figure 6 and Figure S7). A smaller but similar effect was also perceived with genistein, another GLUT1 and GLUT4 inhibitor, which decreased carb-NDI uptake by 43-44% for compounds **5** and **6.** This inhibition was not observed for compound **1** where the carbohydrate is directly linked to the NDI-azide scaffold. Unexpectedly, the presence of the specific GLUT1 inhibitor WZB117 yielded no significant cell uptake differences with the inhibitor-free control for all carb-NDIs examined. In addition, the aglycone-NDI **7** showed a small cell uptake inhibition (8-24%) in the presence of the GLUT inhibitors examined. This small effect has been previously reported for other aglycone-drugs such as cyclohexane-1,2-diamine platinum derivatives when their cell uptake was assayed in presence of phloretin or 4,5-*O*-ethylidene-α-D-glucose GLUT inhibitors.[26b]

10.1002/chem.201604886

HT29-uptake Inhibition (%)

Inhibitor	Glut Target	Prop-NDI **7**	glc-NDI **1**	manC2-NDI **5**	glcC2-NDI **6**
ZWB117	Glut 1	○ 20	○ -19	○ 7	○ 18
Quercetin	Glut 1 & 4	○ 24	● 54	◑ 35	● 64
Genistein	Glut 1 & 4	○ 9	○ 17	● 43	● 44
Ritonavir	Glut 4	○ 8	○ 0	◑ 28	● 59

Inhibition (%)	
% < 25	○ Small
25 > % > 40	◑ Medium
% > 40	● High

Figure 6. Cell uptake inhibition in HT-29 cancer cells of β-glc-NDI **1**, α-man-NDI **5**, β-glcC2-NDI **6** and aglycone-NDI **7** in the presence of several GLUT inhibitors as measured by intracellular concentration using fluorescence spectroscopy. Pre-incubation with inhibitors (100 μM) for 1h was followed by incubation with of NDI-compounds (5 μM) for 2h.

In order to confirm that GLUT4 is playing an important role in the cell uptake of carb-NDIs **5** and **6**, we carried out the cell uptake inhibition assays in the presence of the specific GLUT4 inhibitor ritonavir (Figure 6). Cellular uptakes of compounds **5** and -particularly- **6** decreased, without affecting neither compound **1** nor control aglycone-NDI **7**. Our results point out to the main role of GLUT4 translocating compounds **5** and **6** containing mannose and glucose, respectively, attached through a short linker to the azide-NDI scaffold. GLUT1, which was previously identified as the main glucose transporter involved in cellular uptake of other glucose-conjugated drugs, seems to be playing a minor role, if any, translocating carb-NDIs **5** and **6**. It is important to note that the functional groups, size and shape of the attached drug may also have a strong influence on the interaction with the glucose transporter and its resulting translocation process. Moreover, other GLUT isoforms could also be involved in the cell uptake of these carbohydrate-conjugated G4 ligands being especially pertinent for compound **1** which possesses a glucose directly linked to the azide-NDI structure.

10.1002/chem.201604886

When we examined the influence of GLUT inhibitors in cell uptake of NDI derivatives **1**, **5**, **6** and **7** in non-cancerous MRC-5 cells (Figures S7-S8), we observed no inhibition with any of the GLUT inhibitors tried. The exception was α-manC2-NDI **5**, which was partially inhibited by quercetin and genistein (28-37%). These results seem to correlate with the overexpression of glucose transporters in cell membranes of cancerous cells such as HT-29, in comparison to a smaller expression in non-cancerous cells such as MRC-5.

Confocal microscopy analysis was used to determine the localization of NDI derivatives β-glc-NDI **1**, α-man-NDI **5**, β-glcC2-NDI **6** and aglycone-NDI **7** in HT-29 and MRC-5 cells. After incubation at 37 ºC for 2h, all four compounds were detected inside the cells and were mostly localized in the nucleus (see Figure 7 for α-man-NDI **5** and Figures S8-10).

These experiments confirm that the glycoconjugated NDIs may reach their DNA G-quadruplex target within the nuclear genome. Similar results were found with the control compound **7**, in agreement with the location of other reported NDI derivatives such as Neidle's tetra-charged naphthalene-diimide MM41.[18, 20] It is important to note that our carbohydrate modified NDIs possess only two positive charged groups and still locate at the nucleus.

10.1002/chem.201604886

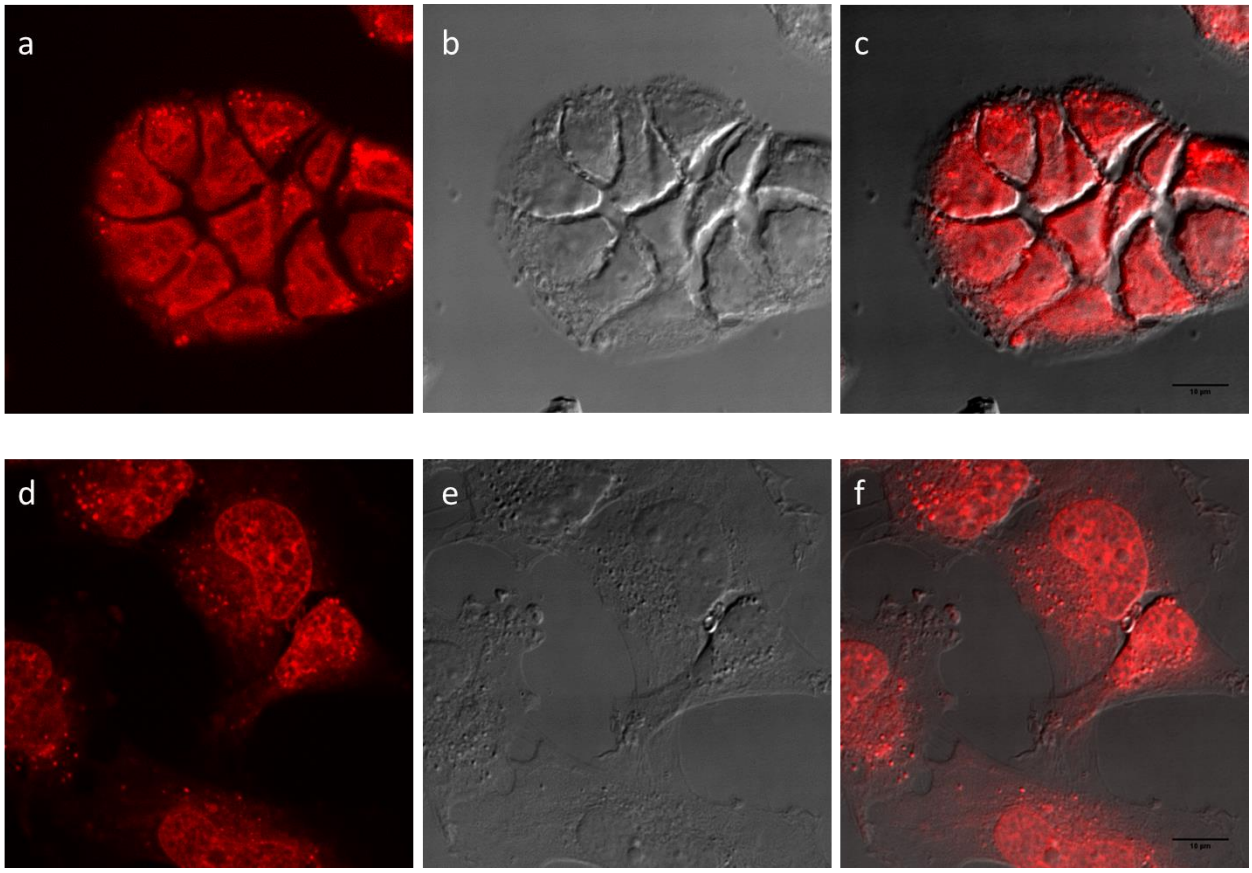


Figure 7. Confocal images of HT-29 and MRC-5 cells after co-incubation with α-man-NDI **5** (5 μM) for 2h. HT-29 cells: a) fluorescence image of compound **5**; b) Transmitted light image (DIC); c) merged image; MRC-5 cells: d) fluorescence image of compound **5**; e) Transmitted light image (DIC); f) merged image.

In vitro antiproliferative studies. The *in vitro* antiproliferative activity of compounds **1**-**7** was measured using the MTT methodology against three cancer cell lines, HT-29, MCF-7 and HeLa (cervical cancer) together with a non-cancerous cell line, MRC-5 (Table 2). Aglycone-NDI **7** with no carbohydrate in its structure displayed the lowest IC50 values in all cancerous cell lines (0.13-0.29 μM) closely followed by ethyl-linked mannose and glucose carb-NDIs, **5** and **6** (0.15-

10.1002/chem.201604886

0.42 μM) and spacer-free 6-deoxyglucose carb-NDI **3** (0.35-0.69 μM). Carb-NDIs (**1**, **3**, **5** and **6**) and control **7** displayed less toxicity for the non-cancerous cell line within a factor of 1.4-6.5 fold. Moreover, the presence of the carbohydrate reduced the toxicity in MRC-5 cells (up to 5- fold for β-malt-NDI, **4**) when compared to aglycone-NDI **7**, the most toxic compound of the series.

Whereas important cell uptake differences were observed among control aglycone NDI **7** and the glycoconjugated NDI derivatives **1**-**6**, there is no a clear correlation with the IC50 values found. However, when comparing the sugar-derived NDIs, some correlation is observed. Cell uptake of β-glcC2-NDI **6** into HT-29 and MCF-7 cells, which was 2-3 fold more efficient than for β-glc-NDI **1**, correlates with its 5.7-8 fold higher cytotoxicity IC_{50} values.

Table 2. IC_{50} values (μM ± SD) of compounds **1**-**7**.

Compound	HT-29	MCF-7	HeLa	MRC-5
1 β-glc-NDI	2.92 ± 0.48	1.19 ± 0.31	1.56 ± 0.70	1.15 ± 0.29
2 β-glcNAc-NDI	2.26 ± 1.03	1.06 ± 0.01	0.95 ± 0.76	0.51 ± 0.01
3 β-6dglc-NDI	0.40 ± 0.08	0.69 ± 0.71	0.35 ± 0.05	0.91 ± 0.32
4 β-malt-NDI	1.85 ± 0.19	1.42 ± 0.38	0.54 ± 0.34	2.04 ± 0.05
5 α-manC2-NDI	0.42 ± 0.05	0.15 ± 0.04	0.29 ± 0.14	0.81 ± 0.44
6 β-glcC2-NDI	0.36 ± 0.13	0.24 ± 0.16	0.24 ± 0.03	0.71 ± 0.25
7 NDI-prop	0.12 ± 0.02	0.13 ± 0.07	0.29 ± 0.18	0.36 ± 0.16

Conclusions.

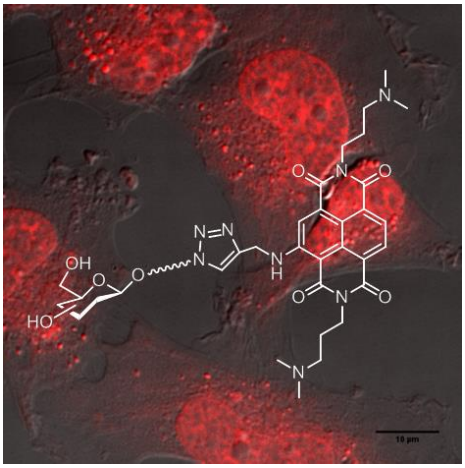
Novel carb-NDIs **1**-**6** were designed and synthesized. We have observed that the presence of the sugar in carb-NDIs is not detrimental for G4 binding and they showed some selectivity against DNA duplexes. However, no clear selectivity for a given G4 topology was observed although compounds **1**-**6** showed higher stabilization of the polymorphic human telomere G4 structures. Moreover, the different sugar units affected the global G4 stabilisation without changing the overall selectivity profile. Cell uptake studies revealed a fast cell penetration of aglycone NDI **7** in a non-temperature-dependent manner whereas carb-NDIs uptake mechanism was found to be very different, being mainly transported through GLUT and dependent on time, temperature, type of carbohydrate and sugar accessibility. Additionally, carb-NDI cell uptake correlated to some extent with their cytotoxicity properties, as seen for β-glcC2-NDI **6,** which entered the cells more efficiently and was significantly more toxic in both HT-29 and MCF-7 cells than β-glc-NDI **1**. Selectivity towards cancerous cell lines also seems dependent on sugar type and attachment, resulting in punctual selectivity gain when compared to that of aglycone-NDI **7**. Noticeably, cell uptake studies in the presence of a variety of GLUT inhibitors showed that α-manC2-NDI **5** and β-glcC2-NDI **6** are transported at least partially through GLUT4. Finally, confocal microscopy studies confirmed that carb-NDIs can reach the nucleus where their intended genomic G4 DNA targets are located.

Acknowledgements.

Financial support from the Spanish Ministerio de Economía y Competitividad (CTQ2012-35360, CTQ2015-64275-P and SAF2016-80228-R), Junta de Andalucía (BIO1786), Worldwide Cancer Research Foundation (16-0290), Italian Association for Cancer Research (AIRC, IG2013-14708),

Agence Nationale de la Recherche (ANR Quarpdiem, ANR-12-BSV8-0008-01) and FEDER funds from the EU are gratefully acknowledged. M.A.R. thanks Ministerio de Educación for a FPU predoctoral fellowship. A.D.R. is the recipient of an ANRS postdoctoral fellowship. We also thank Aurore Guédin for helpful advice.

Graphical Abstract



Carbohydrate naphthalene diimide conjugates (carb-NDIs) have been designed and prepared as G-quadruplex (G4) ligands. Carb-NDIs showed different cell uptake depending on the sugar attachment to the central aromatic core. In fact, a more efficient cell penetration by carb-NDIs correlated with their improved cytotoxic activity in cancer cells. Glucose transporters participate in the specific translocation of carb-NDIs, particularly GLUT4.

Literature

[1] J. T. Davis, *Angew. Chem.* **2004**, 116, 684-716; *Angew. Chem. Int. Ed. Engl.* **2004**, *43*, 668-698.
[2] S. Balasubramanian, L. H. Hurley and S. Neidle, *Nat Rev Drug Discov* **2011**, *10*, 261-275.
[3] a) K. Zhang, C. J. Donnelly, A. R. Haeusler, J. C. Grima, J. B. Machamer, P. Steinwald, E. L. Daley, S. J. Miller, K. M. Cunningham, S. Vidensky, S. Gupta, M. A. Thomas, I. Hong, S. L. Chiu, R. L. Huganir, L. W. Ostrow, M. J. Matunis, J. Wang, R. Sattler, T. E. Lloyd and J. D. Rothstein, *Nature* **2015**, *525*, 56-61; b) A. Kovanda, M. Zalar, P. Sket, J. Plavec and B. Rogelj, *Sci Rep* **2015**, *5*, 17944.
[4] a) R. Perrone, M. Nadai, I. Frasson, J. A. Poe, E. Butovskaya, T. E. Smithgall, M. Palumbo, G. Palu and S. N. Richter, *J Med Chem* **2013**, *56*, 6521-6530; b) A. Rajendran, M. Endo, K. Hidaka, P. L. Tran, J. L. Mergny, R. J. Gorelick and H. Sugiyama, *J Am Chem Soc* **2013**, *135*, 18575-18585; c) S. Amrane, A. Kerkour, A. Bedrat, B. Vialet, M. L. Andreola and J. L. Mergny, *J Am Chem Soc* **2014**, *136*, 5249-5252; d) S. Artusi, M. Nadai, R. Perrone, M. A. Biasolo, G. Palu, L. Flamand, A. Calistri and S. N. Richter, *Antiviral Res* **2015**, *118*, 123-131.
[5] S. A. Ohnmacht and S. Neidle, *Bioorg Med Chem Lett* **2014**, *24*, 2602-2612.
[6] a) R. Lucas, I. Gómez-Pinto, A. Aviñó, J. J. Reina, R. Eritja, C. González and J. C. Morales, *J. Am. Chem. Soc.* **2011**, *133*, 1909-1916; b) R. Lucas, E. Vengut-Climent, I. Gómez-Pinto, A. Aviñó, R. Eritja, C. González and J. C. Morales, *Chem. Commun.* **2012**, *48*, 2991-2993.
[7] I. Gomez-Pinto, E. Vengut-Climent, R. Lucas, A. Aviñó, R. Eritja, C. Gonzalez and J. C. Morales, *Chem. Eur. J.* **2013**, *19*, 1920-1927.
[8] M. Di Antonio, G. Biffi, A. Mariani, E. A. Raiber, R. Rodriguez and S. Balasubramanian, *Angew. Chem.* 2012, 124, 11235-11240; *Angew. Chem. Int. Ed. Engl.* **2012**, *51*, 11073-11078.
[9] J. L. Zhang, Y. Fu, L. Zheng, W. Li, H. Li, Q. Sun, Y. Xiao and F. Geng, *Nucleic Acids Res* **2009**, *37*, 2471-2482.
[10] K. Duskova, S. Sierra, M. S. Arias-Perez and L. Gude, *Bioorg Med Chem* **2016**, *24*, 33-41.
[11] a) M. Kaiser, A. De Cian, M. Sainlos, C. Renner, J. L. Mergny and M. P. Teulade-Fichou, *Org Biomol Chem* **2006**, *4*, 1049-1057; b) L. Xue, N. Ranjan and D. P. Arya, *Biochemistry* **2011**, *50*, 2838-2849; c) N. Ranjan, E. Davis, L. Xue and D. P. Arya, *Chem Commun (Camb)* **2013**, *49*, 5796-5798.
[12] a) F. Doria, I. Manet, V. Grande, S. Monti and M. Freccero, *J. Org. Chem.* **2013**, *78*, 8065-8073; b) F. Doria, C. M. Gallati and M. Freccero, *Org Biomol Chem* **2013**, *11*, 7838-7842.
[13] a) F. Cuenca, O. Greciano, M. Gunaratnam, S. Haider, D. Munnur, R. Nanjunda, W. D. Wilson and S. Neidle, *Bioorg Med Chem Lett* **2008**, *18*, 1668-1673; b) R. Perrone, F. Doria, E. Butovskaya, I. Frasson, S. Botti, M. Scalabrin, S. Lago, V. Grande, M. Nadai, M. Freccero and S. N. Richter, *J Med Chem* **2015**, *58*, 9639-9652; c) M. Micco, G. W. Collie, A. G. Dale, S. A. Ohnmacht, I. Pazitna, M. Gunaratnam, A. P. Reszka and S. Neidle, *J Med Chem* **2013**, *56*, 2959-2974.
[14] a) F. Doria, A. Oppi, F. Manoli, S. Botti, N. Kandoth, V. Grande, I. Manet and M. Freccero, *Chem Commun (Camb)* **2015**, *51*, 9105-9108; b) M. Zuffo, F. Doria, V. Spalluto, S. Ladame and M. Freccero, *Chem. Eur. J.* **2015**, *21*, 17596-17600; c) F. Doria, M. Folini, V. Grande, G. Cimino-Reale, N. Zaffaroni and M. Freccero, *Org Biomol Chem* **2015**, *13*, 570-576.
[15] F. Doria, M. Nadai, M. Folini, M. Scalabrin, L. Germani, G. Sattin, M. Mella, M. Palumbo, N. Zaffaroni, D. Fabris, M. Freccero and S. N. Richter, *Chem. Eur. J.* **2013**, *19*, 78-81.
[16] M. Nadai, F. Doria, L. Germani, S. N. Richter and M. Freccero, *Chem. Eur. J.* **2015**, *21*, 2330-2334.
[17] A. Lopergolo, R. Perrone, M. Tortoreto, F. Doria, G. L. Beretta, V. Zuco, M. Freccero, M. Grazia Borrello, C. Lanzi, S. N. Richter, N. Zaffaroni and M. Folini, *Oncotarget* **2016**, *7*, 49649-49663.
[18] E. Salvati, F. Doria, F. Manoli, C. D'Angelo, A. Biroccio, M. Freccero and I. Manet, *Org Biomol Chem* **2016**, *14*, 7238-7249.
[19] G. W. Collie, R. Promontorio, S. M. Hampel, M. Micco, S. Neidle and G. N. Parkinson, *J Am Chem Soc* **2012**, *134*, 2723-2731.
[20] S. A. Ohnmacht, C. Marchetti, M. Gunaratnam, R. J. Besser, S. M. Haider, G. Di Vita, H. L. Lowe, M. Mellinas-Gomez, S. Diocou, M. Robson, J. Šponer, B. Islam, R. Barbara Pedley, J. A. Hartley and S. Neidle, *Sci. Rep.* **2015**, *5*, 11385.

[21] M. Gunaratnam, M. d. l. Fuente, S. M. Hampel, A. K. Todd, A. P. Reszka, A. Schätzlein and S. Neidle, *Bioorg Med Chem* **2011**, *19*, 7151-7157.
[22] a) E. C. Calvaresi and P. J. Hergenrother, *Chem Sci* **2013**, *4*, 2319-2333; b) C. Granchi, S. Fortunato and F. Minutolo, *Medchemcomm* **2016**, *7*, 1716-1729.
[23] D. Z. Liu, S. Sinchaikul, P. V. Reddy, M. Y. Chang and S. T. Chen, *Bioorg Med Chem Lett* **2007**, *17*, 617-620.
[24] J. Cao, S. Cui, S. Li, C. Du, J. Tian, S. Wan, Z. Qian, Y. Gu, W. R. Chen and G. Wang, *Cancer Res* **2013**, *73*, 1362-1373.
[25] E. C. Calvaresi, C. Granchi, T. Tuccinardi, V. Di Bussolo, R. W. Huigens, 3rd, H. Y. Lee, R. Palchaudhuri, M. Macchia, A. Martinelli, F. Minutolo and P. J. Hergenrother, *Chembiochem* **2013**, *14*, 2263-2267.
[26] a) P. Liu, Y. Lu, X. Gao, R. Liu, D. Zhang-Negrerie, Y. Shi, Y. Wang, S. Wang and Q. Gao, *Chem Commun (Camb)* **2013**, *49*, 2421-2423; b) M. Patra, T. C. Johnstone, K. Suntharalingam and S. J. Lippard, *Angew Chem Int Ed Engl* **2016**, *55*, 2550-2554; c) M. Patra, S. G. Awuah and S. J. Lippard, *J Am Chem Soc* **2016**.
[27] J. Pohl, B. Bertram, P. Hilgard, M. R. Nowrousian, J. Stuben and M. Wiessler, *Cancer Chemother Pharmacol* **1995**, *35*, 364-370.
[28] a) H. Miyake, C. Otsuka, S. Nishimura and Y. Nitta, *J Biochem* **2002**, *131*, 587-591; b) E. D. Soli, A. S. Manoso, M. C. Patterson, P. DeShong, D. A. Favor, R. Hirschmann and A. B. Smith, *J. Org. Chem.* **1999**, *64*, 3171-3177.
[29] P. Quagliotto, G. Viscardi, C. Barolo, D. D'Angelo, E. Barni, C. Compari, E. Duce and E. Fisicaro, *J. Org. Chem.* **2005**, *70*, 9857-9866.
[30] A. De Cian, L. Guittat, M. Kaiser, B. Saccà, S. Amrane, A. Bourdoncle, P. Alberti, M.-P. Teulade-Fichou, L. Lacroix and J.-L. Mergny, *Methods* **2007**, *42*, 183-195.
[31] F. Doria, M. Nadai, M. Folini, M. Di Antonio, L. Germani, C. Percivalle, C. Sissi, N. Zaffaroni, S. Alcaro, A. Artese, S. N. Richter and M. Freccero, *Org Biomol Chem* **2012**, *10*, 2798-2806.
[32] a) Y. Wang and D. J. Patel, *Structure* **1994**, *2*, 1141-1156; b) K. W. Lim, V. C. M. Ng, N. Martín-Pintado, B. Heddi and A. T. Phan, *Nucleic Acids Res* **2013**, *41*, 10556-10562.
[33] a) G. N. Parkinson, M. P. H. Lee and S. Neidle, *Nature* **2002**, *417*, 876-880; b) B. Heddi and A. T. Phan, *J Am Chem Soc* **2011**, *133*, 9824-9833.
[34] K. W. Lim, S. Amrane, S. Bouaziz, W. Xu, Y. Mu, D. J. Patel, K. N. Luu and A. T. Phan, *J Am Chem Soc* **2009**, *131*, 4301-4309.
[35] a) K. N. Luu, A. T. Phan, V. Kuryavyi, L. Lacroix and D. J. Patel, *J Am Chem Soc* **2006**, *128*, 9963-9970; b) A. T. Phan, V. Kuryavyi, K. N. Luu and D. J. Patel, *Nucleic Acids Res* **2007**, *35*, 6517-6525.
[36] A. De Rache and J. L. Mergny, *Biochimie* **2015**, *115*, 194-202.
[37] F. Doria, M. Nadai, M. Folini, M. Di Antonio, L. Germani, C. Percivalle, C. Sissi, N. Zaffaroni, S. Alcaro, A. Artese, S. N. Richter and M. Freccero, *Org Biomol Chem* **2012**, *10*, 2798-2806.
[38] Y. Liu, Y. Cao, W. Zhang, S. Bergmeier, Y. Qian, H. Akbar, R. Colvin, J. Ding, L. Tong, S. Wu, J. Hines and X. Chen, *Mol Cancer Ther* **2012**, *11*, 1672-1682.
[39] A. K. Vyas, J. C. Koster, A. Tzekov and P. W. Hruz, *J Biol Chem* **2010**, *285*, 36395-36400.
[40] P. Strobel, C. Allard, T. Perez-Acle, R. Calderon, R. Aldunate and F. Leighton, *Biochem J* **2005**, *386*, 471-478.