An Acyclic Oligoheteroaryle That Discriminates Strongly between Diverse G-Quadruplex Topologies**

Florian Hamon, Eric Largy, Aurore Guédin-Beaurepaire, Myriam Rouchon-Dagois, Assitan Sidibe, David Monchaud, Jean-Louis Mergny, Jean-François Riou, Chi-Hung Nguyen, and Marie-Paule Teulade-Fichou*

Quadruplex nucleic acids are secondary structures that may form in sequences containing guanine repeats.[1] These structures are strongly suspected to interfere with the transfer and the maintenance of the genetic information and therefore are the focus of considerable attention.[2] Quadruplexes may accommodate small synthetic compounds that could be used as probes to decipher their functions or as pharmacological agents to block various vital functions at the level of DNA or RNA.[3] These synthetic ligands should fulfill an essential requirement to enable correlating their in cellulo biological effects to their quadruplex recognition ability, namely strong specificity for the targeted quadruplex combined with poor association to duplex DNA (ideally a difference of two orders of magnitude or more between the affinity constants is desired).[4] Among the large number of quadruplex binders reported to date, the first one having met these criteria is telomestatin, a natural oxazole-based macrocycle (Scheme 1).[5] Telomestatin is neutral, thus differentiating it from the vast majority of G-quadruplex binders that display fused aromatic skeletons with cationic charges.[3a] Overall the macrocyclic shape of telomestatin is recognized to dominate the interaction with quadruplex structures and to be responsible for its absence of affinity for duplex DNA.[6] However, two recent studies have shown that cations may be involved in the binding of telomestatin with quadruplex DNA, thus suggesting the existence of a multivalent interaction that is more complex than anticipated.[7] Up to recently, telomestatin remained accessible by an arduous multistep synthetic pathway.[8] Although this method has been significantly optimized,[9] the impressive antitumor activity of this natural product[10] has prompted efforts to develop oxazole-based macrocyclic analogues.[11] These synthetic macrocycles elicit quadruplex-interacting properties, but most of them require derivatization with cationic linkers to be active, which demonstrates that the macrocyclic shape is the essential determinant of selectivity.

To address this question of importance in the establishment of G-quadruplex recognition rules, we were keen to develop further the class of neutral oxazole-based quadruplex binders with a new family that features a nonmacrocyclic oligomeric scaffold with alternate oxazole and pyridine motifs (Scheme 1). In this series, the heptacyclic derivative TOxaPy was found to be totally devoid of affinity for duplex DNA while exhibiting an unprecedented binding preference for certain quadruplex topologies over others. In particular and remarkably, the new compound recognizes exclusively the human telomeric quadruplex in Na\(^+\)-rich buffer and is not active in K\(^+\)-rich buffer (see Figure S1 in the Supporting Information). This unique quadruplex binding profile is strongly dependent on the size of the oligomer, as the...
pentacyclic analogue BOxaPy does not associate to quadruplexes, and may result from groove interactions.

To achieve the synthesis of oligo-heteroaryles, we adopted a convergent procedure based on the cross-coupling of 2,6-bis(oxazol-5-yl)pyridine (I) with 2-bromopyridine derivatives by the double C–H activation of the oxazole rings (Scheme 2).[12] Precursor 1 was obtained in two steps by the oxidation of 2,6-lutidine with selenium dioxide to afford pyridine-2,6-dicarbaldehyde,[13] which was submitted to Van Leuven conditions (see Scheme S1 in the Supporting Information). The coupling of I under classical conditions with two molar equivalents of 2-bromopyridine led to the pentaheteroaryle BOxaPy (bisoxazoletrispyridine). The same coupling reaction was carried out using 2-bromo-5-(pyridine-2-yl)oxazole to prepare the heptaaryle homologue TOxaPy (tetraoxazoletrispyridine; structure shown in Scheme 3).

However, in this case the reaction product was found to be a mixture of TOxaPy and a geometric isomer differing only by one pyridine–oxazole junction (ca. 50:50 from HPLC analysis, Scheme S2 and Figure S2). This mixture results from two equivalent positions available for the second condensation as demonstrated by a deuteriation experiment (see Scheme S3 and Figure S3). To obtain TOxaPy unambiguously, a new route was devised in which terminal oxazole moieties were synthesized from the corresponding pentacyclic bisformyl (Scheme 3). In this approach, I was coupled with 2-bromo-5-(pyridine-2-yl)oxazole to prepare the heptaaryle homologue TOxaPy (tetraoxazoletrispyridine; structure shown in Scheme 3).

Scheme 2. First approach affording BOxaPy and TOxaPy as a mixture of isomers. a) TosMIC, K2CO3, MeOH, 50°C, 16 h (50%); b) CuI, Pd(OAc)2, Cs2CO3, PCy3·HBF4, dioxane, 130°C, 24 h (45%); TosMIC = p-toluenesulfonylmethyl isocyanide; Cy = cyclohexyl.

Scheme 3. Regiospecific synthesis of TOxaPy a) CuI, Pd(OAc)2, Cs2CO3, PCy3·HBF4, dioxane, 130°C, 16 h (50%); b) CuI, Pd(OAc)2, Cs2CO3, PCy3·HBF4, dioxane, 130°C, 24 h (45%); TosMIC = p-toluenesulfonylmethyl isocyanide; Cy = cyclohexyl.

Figure 1. a) Left: Stabilization of F21T (0.2 μm) by TOxaPy (1 μm) alone (black bar) and in presence of ds26 (3 and 10 μm, dark and light gray bars) in Na+ or K+-rich buffer. Right: stabilization of F21T by BOxaPy (1 μm) in the indicated cationic conditions. b) Stabilization of F21T as a function of TOxaPy concentration in Na+ (●) and K+-rich (○) buffer (composition of the Na+- and K+-rich buffers is specified in the Supporting Information). The y axis label in (a) also corresponds to (b).

The ability of the obtained oligomeric compounds to interact with the human telomeric quadruplex was first investigated by a FRET-melting assay (FRET = fluorescence resonance energy transfer) using the doubly labeled sequence F21T [FAM-G3(T2AG3)3-Tamra].[14] Remarkably, TOxaPy induces a large stabilization of F21T in Na+ conditions with ΔT1/2 = 10.8°C (Figure 1a), whereas, in stark contrast, no significant stabilization was observed in K+ conditions (ΔT1/2 < 1.0°C). A concentration-dependent FRET-melting experiment confirmed both the strong stabilizing ability of TOxaPy in Na+ (Figure 1b) and the poor effect in K+ (around +7°C at 10 μM). This behavior is remarkable as all known quadruplex binders exhibit no marked cation-dependence or, if so, show a weaker association in K+-rich buffer (see Figure S4).[14]

Finally, melting experiments indicate that the heptacyclic compound exhibits a very poor ability to associate to duplex DNA, since the stabilization of F21T is only marginally affected by the presence of the 26 base pair duplex competitor ds26 (Figure 1a left panel and Table S1). In addition, the shorter pentacyclic analogue BOxaPy appears unable to stabilize F21T irrespective of the cationic conditions (Figure 1a, right panel).

Taken together, these results suggest that the neutral compound TOxaPy binds exclusively the telomeric quadruplex architecture(s) in sodium conditions and that this unprecedented behavior is strongly dependent on the size of the oligomeric scaffold, which plays a key role in the strength of the interaction.

We next conducted several experiments with TOxaPy to have a deeper insight into this unprecedented binding behavior. Interestingly, TOxaPy is characterized by an intense

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blue fluorescence in water (fluorescence quantum yield $\Phi_F = 0.5$) that is strongly quenched in the presence of DNA. Hence, fluorimetric titrations were conducted with the quadruplex-forming oligonucleotide 22AG [$5^\prime$-AG$_2$(T$_2$AG$_3$)$_4$-3'] and the resulting curves fully confirmed the much stronger binding of TOxaPy in Na$^+$ as compared to K$^+$ conditions (Figure 2 and Figure S5). Fitting of the titration curve in Na$^+$ indicates 1:1 binding stoichiometry with a $K_D$ value in the submicromolar range ($K_D = 2 \times 10^{-3}$ M$^{-1}$), whereas, in the case of K$^+$, the flat shape of the curve is clearly indicative of a low-binding interaction.

Since oxazoles may in principle coordinate to alkali metal cations, it was tempting to attribute the observed effect to the formation of a TOxaPy/Na$^+$ complex prone to interact with the quadruplex, or conversely to a TOxaPy/K$^+$ complex unfavorable to the interaction. However, direct coordination could not be observed by standard spectroscopic measurements as neither the absorption nor the fluorescence of the ligand was affected by large excess of both cations (data not shown). Therefore, fluorimetric titrations were conducted with two quadruplexes whose conformation is not dependent on the bound cation, namely the tetramolecular quadruplex with two quadruplexes whose conformation is not dependent on the cation, thereby indicating a binding mode rather different from the classical $\pi$-stacking on external quartets.

To gain understanding into the surprising structural preferences of TOxaPy, G4-FID titrations were performed using the same DNA sequences. This assay is based on the competitive displacement of the fluorescent light-up probe thiazole orange (TO) by a putative ligand. The ligand association to a given DNA matrix results in fluorescence quenching of TO that reflects the binding affinity, the latter being quantified by the ligand concentration inducing 50% probe displacement ($G_4$D$_{50}$).

The results of the G4-FID assay are shown in Figure 3. Clearly, the probe is strongly displaced in Na$^+$ conditions, whereas it is not in K$^+$ conditions or when the assay is carried out with TBA or the control duplex ds26. However, TOxaPy is not able to displace completely the fluorescent marker, since the fluorescence decrease levels off at 60%, thus preventing determination of $G_4$D$_{50}$, which has poor significance in such case. Conversely, the partial displacement of TO strongly suggests that the competition might be indirect as a result of binding of TOxaPy to a site different from that of TO (loop, groove), thereby indicating a binding mode rather different from the classical $\pi$-stacking on external quartets. Since TO is poorly fluorescent in the tetramolecular quadruplex matrix, the latter was not evaluated by the same assay, but instead other well-known quadruplexes, that is, c-myc$^{[16]}$ and c-kit$^{[17]}$ were tested. Very interestingly, once again, we found that TOxaPy hardly displaces TO from these matrices (Figure S7). The poor binding ability of the ligand for these two quadruplexes was confirmed by very moderate stabilization measured by FRET-melting studies ($\Delta T_{\text{melt}} \approx 5^\circ$C in both cases; see Table S2).

On the whole, the results of the three assays are fully consistent, indicating the strong recognition of the Na$^+$ conformation of the telomeric sequence and the inability of TOxaPy to bind the K$^+$ forms of the human telomeric quadruplex, the antiparallel TBA, and duplex DNA and the moderate recognition of c-myc and c-kit.$^2$

Owing to its oligomeric structure TOxaPy has a high degree of flexibility and thus has the potential to adopt very diverse conformations, enabling adaptation to the geometric constraints of its DNA target. It cannot be excluded that this compound adopts a planar cyclic shape suitable for $\pi$-stacking on quartets (represented in Scheme 1) and that this structural organization may occur inside the quadruplex and be

![Figure 2](image-url)  
*Figure 2.* Fluorimetric titration of TOxaPy (0.5 μM, $\lambda_{ex}$: 340 nm) with 22AG. Plot of the fluorescence area enhancement $F/F_0$ (350–650 nm) as a function of added DNA concentration in Na$^+$ (●) or K$^+$-rich (○) buffer conditions.

![Figure 3](image-url)  
*Figure 3.* Plot of TO displacement vs. TOxaPy concentration with quadruplex DNA (22AG) in Na$^+$ (●) or in K$^+$-rich (○) buffer; TBA in Na$^+$ (●) and in K$^+$-rich (○) buffer, duplex ds26 (●) in K$^+$-rich buffer; [DNA] = 0.25 μM, TO = 0.5 μM with 22AG and TBA, and 0.75 μM with ds26.
mediated by Na\(^+\), as was recently shown for telomestatin. However, this would not be consistent with the strong binding of [TG,T] and the absence of binding of TBA, that both offer accessible external quartets prone to accommodate planar quadruplex binders. It is thus more likely that TOxaPy displays a more or less extended conformation that will favor a nontypical binding mode, presumably through interaction in grooves. Indeed, the latter provide hydrophobic pockets for ribbon-like oligomeric molecules, as shown recently.\(^{[18]}\) This hypothetic binding mode (represented in Figure S8) would explain the absence of binding to TBA that display short grooves.\(^{[19]}\) As well, the absence of binding to the K\(^+\) telomeric form, TBA, could explain the absence of binding to TBA that display short inaccessible grooves, as is the case of the propeller parallel basket-type two-quartet structure \(^{[21]}\) (Figure S1).

With these results in hand, we decided to evaluate the two oligoheteroaryles for their activity on the growth of several cancer cell lines. Remarkably, TOxaPy was found to inhibit strongly the proliferation of the cell lines with IC\(_{50}\) values (concentration required to inhibit 50 % of the growth) down to the nanomolar range (Tables S3 and S4). Conversely, the pentacyclic analogue B0xaPy was poorly efficient even at high concentration (Table S3). This difference in activity is fully consistent with that observed in the FRET-melting assay, thereby underlying the crucial importance of the size of the oligomeric scaffold for both the quadruplex affinity in vitro and the cellular effects. Finally, these results indicate the efficient cellular uptake of TOxaPy and demonstrate its pharmacological potential, which may represent a basis for further developments in anticancer drug discovery.

In summary, using a straightforward and readily scalable synthetic pathway, we expanded the class of oxazolo-based quadruplex ligands and showed the potential of acyclic derivatives for binding quadruplexes with a high and unprecedented specificity. Our findings evidence that a macrocyclic shape is not the only determinant to abolish duplex binding and that acyclic flexible oligomeric scaffolds may adapt specifically to quadruplexes. Importantly, the inability of certain quadruplexes (the K\(^+\) telomeric form, TBA) to accommodate TOxaPy is highly indicative of differences in site accessibility, thereby suggesting the existence of hydrophobic pockets that could be used to anchor ligands specifically. The quadruplex recognition properties of TOxaPy will be further evaluated with the aim of understanding more in depth its interaction with quadruplexes.

Keywords: DNA - G-quadruplexes - heterocycles - molecular recognition


Supporting Information

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# Supporting information

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**Figure S1**: Schematic structure of G-quadruplexes. (A) to (E): human telomeric structures. (A) Propeller-type form observed for d[A(GGGTTA)₃GGG] in a K⁺-containing crystal; (B) (3 + 1) Form 1 observed for d[TA(GGGTTA)₂GGG] in K⁺ solution; (C) (3 + 1) Form 2 observed for d[TA(GGGTTA)₂GGGT] in K⁺ solution; (D) Basket-type form observed for d[A(GGGTTA)₃GGG] in Na⁺ solution; (E) Basket-type form observed for d[[GGGTTA]₂GGGT] in K⁺ solution. (F) Structure of TBA observed for d[GGTTGGTGTTG] in K⁺ solution. (G) Propeller-type form observed for c-kit2 d[GGGCGGGCGCTAGGGAGGGT] in K⁺ solution. (H) Propeller-type form observed for c-myc 2345 d[TGAGGGTGTTAGGGTGGGTAA] in K⁺ solution. (I) Tetrameric parallel-stranded G-quadruplex observed for the TG₃T repeat. anti guanines are plain rectangles; syn guanines are dashed rectangles; loops are colored red. Guanines are purple, Adenines green, Thymine yellow and Cytosine grey.
Synthesis and Characterization of Compounds

Materials and Methods:

\(^1\)H and \(^{13}\)C spectra were recorded at 25°C on a Bruker Avance 300 using TMS as internal standard. Deuterated CDCl\(_3\) and DMSO-d\(_6\) were purchased from SDS. The following abbreviations are used: singlet (s), doublet (d), triplet (t) and multiplet (m). Low-resolution mass spectrometry (ESI-MS) was recorded on a micromass ZQ 2000 (waters). High resolution mass spectrometry (ESI-MS) and elemental analyses were provided by the I.C.S.N. (Institut de Chimie des Substances Naturelles, Gif-sur-Yvette). TLC analysis was carried out on silica gel (Merck 60F-254) with visualization at 254 and 366 nm. Melting points were taken on a Kofler melting point apparatus and are uncorrected. Preparative flash chromatography was carried out with Merck silica gel (Si 60, 40-63 µm). Reagents and chemicals were purchased from Sigma-aldrich, Acros or Alfa-aesar unless otherwise stated. Solvents were purchased from SDS. UV-Vis absorption spectra were recorded on a Secoman Uvikon XL spectrophotometer, fluorescence emission spectra were recorded on a Jobin Yvon FluoroMax-3 spectrophotometer at 20°C.

Scheme S1: Synthesis of the intermediate 1.

2,6-pyridinedicarbaldehyde

2,6-Lutidine (12.9 g, 0.12 mol) in 1,2-dichlorobenzene (50 mL) was added at reflux under stirring over a period of 20 min to a mixture of selenium dioxide (40.6 g, 0.37 mol) in the same solvent (250 mL). The heating was pursued during 4 h (because of the incompatibility of o-dichlorobenzene and oxidizing agents\(^1\)), the use of a shield to protect the manipulator is recommended), then cooled to room temperature. Filtration through a celite pad was followed by the addition of 1N hydrochloric acid (3 x 150 mL) to the filtrate. The aqueous phases were collected, washed with dichloromethane (100 mL) and the organic layer was discarded. The aqueous phase was then neutralized by saturated aqueous sodium bicarbonate solution and extracted with dichloromethane (3 x 150 mL). The organic layers were dried over magnesium sulphate and the solvent was evaporated at reduced pressure. After purification by column chromatography as described above, the pure product was isolated (8.3 g, 50%) which is identical to that described by Swern oxidation method. \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 10.1 (s, 2H), 8.30 (t, \(^3\)J(H,H)=7.2 Hz, 1H), 8.21 (d, \(^3\)J(H,H)=7.2 Hz, 2H) ppm; \(^{13}\)C NMR (75 MHz, CDCl\(_3\)): \(\delta\) 192.4, 153.0, 138.4, 125.4 ppm; LRMS (ESI-MS): m/z = 214.1 [M+H]+.

2,6-bis(oxazol-5-yl)pyridine 1

In a 250 mL round-bottomed flask, 2,6-pyridine dicarboxaldehyde 5 (4.0 g, 29.2 mmol, 1.0 eq.), toluenesulfonyl-methyl isocyanide (11.4 g, 58.3 mmol, 2.0 eq.) and potassium carbonate (16.3 g, 118 mmol, 4.0 eq.) were dissolved in MeOH (100 mL). The mixture was refluxed for 3h. The solvent was evaporated in vacuum and the residue was poured into brine solution and extracted with CH\(_2\)Cl\(_2\) (4x150 mL). The combined organic layers were dried over MgSO\(_4\) and concentrated under vacuum. The residue was purified by flash chromatography (SiO\(_2\), CH\(_2\)Cl\(_2\)/EtOH, 95:5) to afford 2,6-Bis(oxazol-5-yl)pyridine 1 (5.6 g, 90% yield) as a yellow solid.

m.p.: 190-191°C; \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 7.96 (s, 2H), 7.80 (t, \(^3\)J(H,H)= 8.0 Hz, 1H), 7.74 (s, 2H), 7.56 (d, \(^3\)J(H,H)= 8.0 Hz, 2H) ppm; \(^{13}\)C NMR (75 MHz, CDCl\(_3\)): \(\delta\) = 151.0, 150.8, 147.4, 138.0, 125.7, 118.7 ppm; LRMS (ESI-MS) m/z: 214.1 [M+H]+; Anal. calcd for C\(_{11}\)H\(_7\)N\(_3\)O\(_2\): C, 61.97; H, 3.31; N, 19.71. found: C, 61.51; H, 3.43; N, 19.48.
2,6-bis(2-(pyridin-2-yl)oxazol-5-yl)pyridine BOxaPy

In a 10 mL flame-dried round-bottomed flask flushed with argon, 2,6-bis(oxazol-5-yl)pyridine 1 (100 mg, 0.47 mmol, 1.0 eq.), 2-bromopyridine (0.148 ml, 1.55 mmol, 3.3 eq.), palladium acetate (42.1 mg, 0.19 mmol, 0.4 eq.), cesium carbonate (672 mg, 2.06 mmol, 4.4 eq.), copper(I) iodide (205 mg, 1.08 mmol, 2.2 eq.), 3-(dicyclohexylphosphino)cyclohexan-1-ylidene, BF4− (34.4 mg, 0.094 mmol, 0.2 eq.) were suspended in dioxane (2 ml). The reaction was heated for 24 hours at 130 °C under argon. The black mixture was filtered. The filtrate was evaporated and the residue was purified by flash chromatography (SiO2, CH2Cl2/EtOH: 95/5). The fractions containing the product were combined, evaporated and dissolved in a minimum amount of CH2Cl2 (1-2 ml). Cyclohexane (10 volumes) was added and the mixture was partially reduced in vacuum to half of its volume. The formed precipitate was filtered, washed with cyclohexane and dried under vacuum to afford BOxaPy (81 mg, 47% yield) as a slightly orange powder.

m.p.: 242-245°C; 1H NMR (300 MHz, CDCl3): δ=8.77 (d, 3J(H,H)=4.5Hz, 2H) 8.23 (d, 3J(H,H)=7.9 Hz, 2H), 7.97 (s, 2H), 7.88-7.83 (m, 5H), 7.40 (m, 2H) ppm; 13C NMR (75 MHz, CDCl3): δ=161.5, 152.3, 150.9, 147.9, 146.6, 138.5, 137.8, 128.6, 125.6, 123.3, 119.7 ppm; LRMS [ESI-MS]: m/z=368.0 [M+H]+; Anal. calcd for C21H13N5O2·0.5 H2O: C, 67.02; H, 3.75, found: C, 66.81; H, 4.28.
Scheme S2: First synthetic pathway to synthesize heptaaryl compounds affording an isomer mixture.

2-bromo-5-(pyridin-2-yl)oxazole

In a 250 mL flask, 6-bromopyridine-2-carbaldehyde (4.0 g, 21.5 mmol, 1.0 eq.), toluenesulfonyl-methyl isocyanide (4.2 g, 21.5 mmol, 1.0 eq.) and potassium carbonate (6.0 g, 43.4 mmol, 2.0 eq.) were dissolved in MeOH (75 mL). The mixture was refluxed for 3h. The solvent was evaporated under reduced pressure and the residue was poured into brine solution and extracted with CH$_2$Cl$_2$ (4x100 mL). The organic layers were combined, dried over MgSO$_4$ and concentrated under vacuum. The residue was purified by flash chromatography (SiO$_2$, CH$_2$Cl$_2$/EtOH, 95:5) to afford 2-bromo-5-(pyridin-2-yl)oxazole (2.9 g, 60% yield) as a yellow solid.

m.p.: 90-91°C; $^1$H NMR (300 MHz, CDCl$_3$): $\delta$=7.97 (s, 1H), 7.73 (s, 1H), 7.57 (m, 2H), 7.38 (dd, $^3$J(H,H)= 3.5 & 5.3 Hz, 1H) ppm; $^{13}$C NMR (75 MHz, CDCl$_3$) : $\delta$=151.6, 148.0, 142.4; 139.3, 127.6, 126.4, 118.1 ppm; LRMS (ESI-MS): m/z=225 [M+H]$^+$; Anal. calcd for C$_8$H$_5$BrN$_2$O : C, 42.70%; H, 2.24%; N, 12.45% found: C, 42.71%; H, 2.27%; N, 12.19%. 

Reagents and conditions: (a): $p$-Toluenesulfonylmethyl isocyanide, K$_2$CO$_3$, MeOH, rfx 3 h; (b): Pd(OAc)$_2$, PCy$_3$HBF$_4$, CuI, Cu$_2$CO$_3$, toluene, dioxane, 130°C

Scheme S2: First synthetic pathway to synthesize heptaaryl compounds affording an isomer mixture.
Mixture of TOxaPy and its geometric isomer

BOxaPy synthesis conditions were used starting from 2,6-bis(oxazol-5-yl)pyridine 1 and 2-bromo-5-(pyridin-2-yl)oxazole. Similar reaction conditions afforded a mixture, which was analyzed by LC and LC-MS.

LRMS (ESI-MS, H2O/MeOH): m/z = 534.1 [M+Na]+; HRMS (ESI-MS): 534.1369 (calculated: 534.1390 C27H21N5O6Na)

Figure S2: chromatogramm of the mixture of compounds obtained by the first synthetic pathway. HPLC Gradient H2O/acetonitrile

Deuteration experiments

Reagents and conditions: (a): i) TMEDA, LiHMDS, THF, -78°C, 1h ii) D2O, RT, 20h;
(b): Pd(OAc)2, PCy3, HBF4, Cat, Cs2CO3, toluene, dioxane, 130°C

Scheme S3: Deuteration experiments to confirm isomer structure

2,6-bis-(2-deuterooxazol-5-yl)pyridine 7

In an argon flushed flask, 2,6-bis(oxazol-5-yl)pyridine 1 (100 mg, 0.5 mmol, 1.0 eq.) and TMEDA (0.16 mL, 1.0 mmol, 2.0 eq.) were dissolved in anhydrous THF (5 ml) and cooled to -78°C. LiHMDS (1M in THF, 1.0 mL, 1.0 mmol, 2.0 eq.) was added dropwise and mixture was stirred for 1h at -78°C and 30 min at -40°C. The mixture was then cooled back to -78°C before addition of D2O (34 µL, 1.9 mmol, 3.8 eq.). The mixture was stirred for 1h at -78°C, then for 20h at RT. The reaction mixture was extracted with AcOEt (3x10 mL). The organic layers were combined, dried over
MgSO₄ and evaporated under reduced pressure to afford 2,6-bis-(2-deuterooxazol-5-yl)pyridine (83 mg, 83% yield) as a yellow solid.

**m.p.:** 185-187°C; **¹H NMR** (300 MHz, CDCl₃): δ=7.56 (d, ³J(H,H)= 7.9 Hz, 2H), 7.74 (s, 2H), 7.80 (t, ³J(H,H)=7.9 Hz, 1H) ppm; **¹³C NMR** (75 MHz, CDCl₃) : δ=151.5, 150.8, 147.4, 138.0, 125.7, 118.7 ppm; **LRMS** (ESI-MS): m/z= 216.2 [M+H]^+. 
Mixture of TOxaPy and deuterated TOxaPy isomer

BOxaPy synthesis conditions were used starting from 2,6-bis-(2-deuterooxazol-5-yl)pyridine 6 and 2-Bromo-5-(pyridin-2-yl)oxazole 5. Similar reaction conditions afforded a mixture, which was analyzed by MS. The mass spectrum of the crude mixture showed the formation of the expected TOxaPy (C_{27}H_{15}N_{7}O_{4}Na m/z 524.1) and the deuterated isomer (C_{27}DH_{14}N_{7}O_{4}Na m/z 525.1).

Figure S3: Theoretical mass spectra of TOxaPy and its deuterated analogue. Experimental spectrum recorded with the mixture.
Unambiguous synthetic strategy

2-bromo-6-(1,3-dioxolan-2-yl)pyridine 2

In a 250 ml flask, 6-bromo-2-carbaldehyde pyridine (2 g, 10.75 mmol, 1.0 eq.) and ethylene glycol (1.02 ml, 18.28 mmol, 1.7 eq.) were dissolved in benzene (50 ml). 4-methylbenzenesulfonic acid hydrate (0.10 g, 0.54 mmol, 0.05 eq.) was added and reaction mixture was refluxed under Dean-Stark conditions for 24h. After cooling to RT, mixture was quenched with a 1% aqueous Na₂CO₃ solution. The 2 phases were separated and aqueous phase was extracted with CH₂Cl₂ (2x100 mL). Organic phases were combined, dried over MgSO₄ and concentrated under reduced pressure. The residue was purified by flash chromatography (SiO₂, CH₂Cl₂/EtOH: gradient from 100/0 to 90/10) to afford 2-bromo-6-(1,3-dioxolan-2-yl)pyridine 2 (2.18 g, 88 % yield) as an oil.

1H NMR (300 MHz, CDCl₃): δ=7.60 (t, 3J(H,H)= 7.5 Hz, 1H), 7.52 -7.47 (m, 2H), 5.81 (s, 1H), 4.18 -4.04 (m, 4H) ppm; LRMS (ESI-MS, H₂O/MeOH): m/z = 252 [M+Na]+.

2,6-bis(2-(6-(1,3-dioxolan-2-yl)pyridin-2-yl)oxazol-5-yl)pyridine 3

In a 100 mL flame-dried round-bottomed flask flushed with argon, 2,6-bis(oxazol-5-yl)pyridine (1.47 g, 6.9 mmol, 1,0 eq.), 2-bromo-6-(1,3-dioxolan-2-yl)pyridine (5.36 g, 23.3 mmol, 3.4 eq.), palladium acetate (600 mg, 2.7 mmol, 0.4 eq.), cesium carbonate (9.5 g, 29 mmol, 4.2 eq.), copper(I) iodide (2.9 g, 15 mmol, 2.2 eq.), PCy3•HBF4 (680 mg, 1.8 mmol, 0.3 eq.) were suspended in dioxane (36 ml). The mixture was heated at 130°C for 24h under argon. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography (SiO₂, CH₂Cl₂/EtOH: gradient from 100/0 to 90/10) to afford 2,6-bis(2-(6-(1,3-dioxolan-2-yl)pyridin-2-yl)oxazol-5-yl)pyridine 3 (2.76 g, 78 % yield) as a white powder.

1H NMR (300 MHz, CDCl₃): δ=7.60 (t, 3J(H,H)= 7.5 Hz, 1H), 7.52-7.47 (m, 2H), 5.81 (s, 1H), 4.18-4.04 (m, 4H) ppm; 13C NMR (75 MHz, CDCl₃): δ=160.6, 157.9, 151.6, 147.2, 145.5, 137.9, 137.8, 127.9, 122.8, 121.8, 119.0, 103.6, 65.8 ppm; LRMS (ESI-MS, H₂O/MeOH): m/z= 534.1 [M+Na]+; HRMS (ESI-MS): 534.1369 (calculated: 534.1390 C₂₇H₂₁N₅O₆Na); Anal. calcd for C₂₇H₂₁N₅O₆: C, 62.30; H, 4.26; N, 13.46; found: C, 62.24; H, 4.79; N, 13.19.

6,6’-(5,5’-(pyridine-2,6-diyl)bis(oxazole-5,2-diyl))dipicolinaldehyde 4

In a 50 ml flask, 3 (222 mg, 0.434 mmol) was dissolved in HCl ([2M], 8 mL) and heated to 110°C for 2h. After cooling to RT, mixture was neutralized by addition of saturated NaHCO₃ and extracted with hot CH₂Cl₂ (8x) until complete disappearance of the visible foam at the interface between the 2 phases. The organic phases were combined, dried over MgSO₄, evaporated to afford 6,6’-(5,5’-(pyridine-2,6-diyl)bis(oxazole-5,2-diyl))dipicolinaldehyde 4 (155 mg, 84 % yield) as a white product. 3 was used in the next step without further
purification.

**m.p. > 260°C; **

**H NMR (300 MHz, CDCl₃):** \( \delta = 10.27 \) (s, 2H), 8.50-8.24 (m, 2H), 8.12-8.06 (m, 4H), 8.05 (s, 2H), 7.98-7.94 (m, 1H), 7.92-7.85 (m, 2H) ppm; **LRMS (ESI-MS):** \( m/z = 446.0 \) [M+Na]⁺; **HRMS (ESI-MS):** 446.0882 (calculated: 446.0865 C₂₃H₁₃N₅O₄Na), **Anal. calcd for C₂₃H₁₃N₅O₄:** C, 61.33; H, 3.58; N, 15.55; found: C, 61.39; H, 3.61; N, 15.01.

2,6-bis(2-(6-(oxazol-5-yl)pyridin-2-yl)oxazol-5-yl)pyridine TOxaPy

In a 50 ml flask, 6,6’-(5,5’-(pyridine-2,6-diyl)bis(oxazole-5,2-diyl))dipicolinaldehyde 3 (129 mg, 0.305 mmol, 1.0 eq.), toluene-4-sulfonylmethyl isocyanide (149 mg, 0.762 mmol, 2.5 eq.) and potassium carbonate (211 mg, 1.523 mmol, 5.0 eq.) were dissolved in MeOH (15 ml). The mixture was heated at 50°C for 16h. After cooling to RT, mixture was evaporated and dissolved in AcOEt. The organic phase was extracted with H₂O and brine. The aqueous phases were combined and extracted with AcOEt. The organic phases were combined, dried with MgSO₄ and concentrated under reduced pressure. The residue was purified by flash chromatography (SiO₂, CH₂Cl₂/EtOH: gradient from 100:0 to 93:7) to afford **TOxaPy** (77 mg, 50 % yield) as a white solid.

**m.p. > 260°C; **

**H NMR (300 MHz, CDCl₃):** \( \delta = 8.21 \) (d, \( ^3J(H,H)= 7.2 \) Hz, 2H), 8.05 (s, 2H), 8.02 (s, 2H), 8.00-7.94 (m, 3H), 7.95 (s, 2H), 7.86 (d, \( ^3J(H,H)= 7.9 \) Hz, 2H), 7.80 (d, \( ^3J(H,H)= 7.8 \) Hz, 2H) ppm; **LRMS (ESI-MS):** \( m/z = 524.0 \) [M+Na]⁺; **HRMS (ESI-MS):** 524.1100 (calculated: 524.1083 C₂₇H₁₅N₇O₄Na), **Anal. calcd for C₂₇H₁₅N₇O₄:** C, 64.67; H, 3.02; N, 19.55; found: C, 64.28; H, 2.98; N, 19.18.
FRET-melting:

Stabilization of compounds with quadruplex-structure was monitored via FRET-melting assay, in a version that also enables the determination of the quadruplex- over duplex-DNA selectivity as well as the intra-quadruplex selectivity (De Cian et al, Methods, 2007, 42, 183). FRET-melting assay was performed with oligonucleotides that mimic the human telomeric sequence, as well as other quadruplex-forming oligonucleotides (25Ceb, 21CTA, c-Myc, c-Kit1, c-Kit2) and the control duplex ds26, equipped with FRET partners at each extremities:

- F21T: FAM-G3[T2AG3]3-Tamra,
- FmycT: FAM-T2GAG3TG3TAG3TG3TA2-Tamra,
- FKit1T: FAM-G3AG3CGCTG3AG2AG3-Tamra,
- FKit2T: FAM-G3CG3CGCGAG3AG4-Tamra,
- F25CebT: FAM-AG3TG3TGTA2GTGTG3TG3TA2-Tamra,
- F21CTAT: FAM-G3[CTAG3]3-Tamra,

with FAM: 6-carboxyfluorescein and Tamra: 6-carboxy-tetramethylrhodamine). Measurements were made with excitation at 492 nm and detection at 516 nm. Na⁺ conditions correspond to 10 mM lithium cacodylate pH 7.2 buffer supplemented with 100 mM NaCl; while K⁺ conditions correspond to 10 mM lithium cacodylate pH 7.2 buffer supplemented with 10 mM KCl + 90 mM LiCl.

The stabilisation effect induced by TOxaPy and BOxaPy (1 μM) with the oligonucleotides (0.2 μM) is quantified by the increase in melting temperature (ΔT₁/₂).

<table>
<thead>
<tr>
<th></th>
<th>ΔT₁/₂ (°C)</th>
<th>TOxaPy</th>
<th>BOxaPy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Duplex DNA concentration (ds 26) – (μM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na⁺</td>
<td></td>
<td>10.8</td>
<td>9.4</td>
</tr>
<tr>
<td>K⁺</td>
<td></td>
<td>0.8</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Table S1: Stabilization of F21T by TOxaPy and BOxaPy in presence of increasing concentration of ds26, in both sodium and potassium conditions.
Table S2: Stabilization of various quadruplex-forming oligonucleotides and duplex FdxT by TOxaPy.

<table>
<thead>
<tr>
<th>oligonucleotides</th>
<th>ΔT_{1/2} (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FmycT</td>
<td>5.7</td>
</tr>
<tr>
<td>FKit1T</td>
<td>1.1</td>
</tr>
<tr>
<td>FKit2T</td>
<td>5.5</td>
</tr>
<tr>
<td>FdxT</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Figure S4: Stabilization of F21T (0.2 μM) by well-known quadruplex ligands (indicated) and by quadruplex ligands (non labelled) studied by the co-authorship in previous works. Plot of ΔT_{1/2} values induced in K⁺ and Na⁺- rich buffers. See text reference 1 and references cited therein.
Fluorescence properties of TOxaPy.

Fluorescence titrations and the G4-FID assay were performed in lithium cacodylate buffer solution (10 mM LiAsO$_2$Me$_2$) containing NaCl or KCl (100 mM) and adjusted to pH 7.2 with HCl. The fluorescence spectra were recorded with a HORIBA Jobin-Yvon Fluoromax-3® spectrofluorimeter, in a 3-mL quartz cell (path length: 1 cm) using the following experimental parameters; increment, 1 nm; optical slit widths, 3.0/3.0 nm; integration time, 0.1 s.

Fluorescence titrations were carried out as follows. Fluorescence spectra of TOxaPy alone (0.5 µM; $F_0$) and upon addition of increasing amounts of quadruplex DNA (F) were recorded ($\lambda_{exc}$: 340 nm). The fluorescence enhancement (350–650 nm) was plotted as a function of the concentration of added G4-DNA.

The G4-FID assay was performed in the wavelength range from 510 to 750 nm, with 501 nm as excitation wavelength. The fluorescence spectrum of the buffer solution was recorded and was systematically subtracted from the emission spectra. The solutions of DNA (0.25 µM) were mixed with TO (final concentration for 22AG and ds26: 0.5 and 0.75 µM respectively) and reference fluorescence spectra were recorded. Increasing concentrations (from 0 to 10 molar equivalents, i.e. from 0 to 2.5 µM) of TOxaPy were then added. After a 3-min shaking and equilibration period, a fluorescence spectrum was recorded after each ligand addition step. The percentage of TO displacement is calculated from the fluorescence area variation $FA$ as $TO_D = 100 - [(FA_{x}/FA_0) \times 100]$, where $FA_0$ is the fluorescence area of the reference spectrum. The percentage of displacement is then plotted as a function of the concentration of added compound.

Fluorimetric Titrations:

![Fluorescence spectra](image)

Figure S5: Fluorescence spectra ($\lambda_{exc}$ = 340 nm) of TOxaPy (0.5 µM) upon addition of increasing amounts of 22AG (from 0 to 2.5 µM) in lithium cacodylate 10mM, pH 7.2 + 100mM of NaCl (left) + 100mM KCl (right).
Figure S6: Fluorimetric titration of TOxaPy (0.5 µM) with TBA in lithium cacodylate 10mM, pH 7.2 + 100mM of NaCl (■) or 100 mM KCl (□); or with the tetramolecular quadruplex [TG₃T]₄ in lithium cacodylate 10mM, pH 7.2 + 100mM of NaCl (●) or 100mM KCl (○). Plot of the normalized fluorescence areas (from 350 to 650 nm) against the concentration of added DNA.

Figure S7: G4 FID assay Fluorimetric titration of TOxaPy (0.5 µM) with the c-myc (▲) or c-kit2 (△) oligonucleotides in lithium cacodylate 10mM, pH 7.2 + 100mM of KCl. Plot of the normalized fluorescence areas (from 350 to 650 nm) against the concentration of added DNA.
Antiproliferative activities of TOxaPy.

Cell culture and cell proliferation assay.

The human cell lines were originated from ATCC, except when otherwise stated. The human cell lines KB (month epidermoid carcinoma) and MCR5 (human fetal lung) were grown in D-MEM medium supplemented with 10% fetal calf serum (Invitrogen), in the presence of 100 UI/ml penicilline, 100µg/ml streptomycine and 1.5µg/ml fungizone in 75cm² flask under 5%CO₂, whereas HCT116 (colon adenocarcinoma), HCT15 (colon adenocarcinoma), MCF7 (breast adenocarcinoma), A549 (lung carcinoma), HL60 (promyocytic leukaemia) were grown in RPMI medium. Resistant MCF7 cells were obtained by prolonged treatment with adriamycin. HL60R/R10 were originated from Oncodesign (Dijon, France) and maintained in RPMI medium containing 100nM doxorubicine. Quiescent EPC cells (Epithelioma Papulosum Cyprini) were grown in RPMI without CO₂ at room temperature.

Cells were plated in 96-well tissue culture plates in 200µl medium and treated 24h later with compounds dissolved in DMSO using a Biomek 3000 (Beckman). Controls received the same volume of DMSO (1% final volume). After 72h exposure MTS reagent (Celltiter 96Aqueous One solution, Promega) was added and incubated for 3h at 37°C: the absorbance was monitored at 490nm and results expressed as the inhibition of cell proliferation calculated as the ratio \((1-(OD_{490 \text{ treated}}/OD_{490 \text{ control}}))\times 100\) in triplicate experiments. For IC₅₀ determinations [50% inhibition of cell proliferation], cells were incubated for 72 h following the same protocol with compound concentrations ranged 0.5nM to 10µM in separate duplicate experiments.

The antiproliferative activity of TOxaPy and BOxaPy were determined against a panel of human tumor cell lines. First percentages of inhibition of both compounds were evaluated at a 10⁻⁵M⁻¹ concentration of the ligand. BOxaPy exhibit rather low activity and was not further investigated. TOxaPy appeared to be a much more potent ligand and IC₅₀’s were determined.

<table>
<thead>
<tr>
<th></th>
<th>BOXAPY</th>
<th>TOXAPY</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB</td>
<td>46±3</td>
<td>97±1</td>
</tr>
<tr>
<td>A549</td>
<td>49±1</td>
<td>88±1</td>
</tr>
<tr>
<td>MCF7</td>
<td>0±10</td>
<td>84±2</td>
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<tr>
<td>HL60</td>
<td>28±1</td>
<td>61±3</td>
</tr>
<tr>
<td>HCT116</td>
<td>78±1</td>
<td>nd</td>
</tr>
<tr>
<td>HCT15</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

*Each value was determined in triplicate

Table S3: Percentage of growth inhibition of BOxaPy and TOxaPy at 10⁻⁵M⁻¹ on a panel of human tumor cell lines.
IC50’s of several independent determinations was determined and reported in Table S4. Results indicated that TOxaPy display potent activities ranging from the micromolar to the nanomolar range, excepted for HL60 leukemia where IC50 is >10 µM.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Type</th>
<th>IC50 (µM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB</td>
<td>Nasopharynx carcinoma</td>
<td>0.12/0.11/0.11/0.28</td>
</tr>
<tr>
<td>A549</td>
<td>Lung carcinoma</td>
<td>0.040/0.030</td>
</tr>
<tr>
<td>MCF7</td>
<td>Breast adenocarcinoma</td>
<td>0.91/1.71</td>
</tr>
<tr>
<td>HCT116</td>
<td>Colon carcinoma</td>
<td>0.040/0.055</td>
</tr>
<tr>
<td>HCT15</td>
<td>Colon carcinoma</td>
<td>0.021/0.046</td>
</tr>
<tr>
<td>HL60</td>
<td>Promyelocytic leukaemia</td>
<td>16/30.7</td>
</tr>
</tbody>
</table>

*Each value correspond to independent determination

**Table S4:** Antiproliferative activity of TOxaPy on a panel of human tumor cell lines
Docking

Figure S8: left) possible binding mode of TOxaPy docked in the 143D PDB structure. Docking was performed with Tripos SYBYL-X 1.3. Image was generated with UCSF Chimera[3] and POV-Ray[4]; right) Conformation of TOxaPy bound in the quadruplex structure.