A hydrophilic three side-chained triazatruxene as a new strong and selective G-quadruplex ligand†

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A new hydrosoluble triazatruxene derivative (AZATRUX) is reported to selectively bind to G-quadruplex DNA, as derived by ESI-MS measurements and competition experiments.

Introduction

G-quadruplexes are a family of nucleic acid secondary structures stabilized by G-tetrads, coplanar quartets of guanines held together by a cyclic arrangement of eight unconventional hydrogen bonds (Hoogsteen bonds, Fig. 1).[1] These structures are stabilized by the presence of monovalent cations and the stacking interactions between overlapping G-tetrads: G-quadruplexes can be formed by G-rich strands of DNA as the telomeric sequences.[2] Telomeric single-strand DNA is the substrate of telomerase, an enzyme necessary for telomeric replication, which is over-expressed in most cancer cells and participates in tumor genesis.[3] The formation of a telomeric G-quadruplex blocks telomerase activity and offers an original strategy for new anticancer agents.[4] During the last few years, several families of compounds have been identified which specifically bind to the telomeric quadruplex. These derivatives, called "G-quadruplex DNA ligands", are able to block telomeric replication in cancer cells and to cause replicative senescence and/or apoptosis after a few cell cycles.[5] Crystallographic and molecular modelling studies suggest a ligand/G-quadruplex general model in which ligands interact mainly through terminal π–π stacking at the end of the quadruplex (threading intercalation), without internal intercalation, and by electrostatic interactions of the side chains in the grooves.[6] Classical G-quadruplex ligands, such as substituted acridines, triazines, porphyrins, as well as perylene,[7] coronene[8] and berberine[9] derivatives, present an aromatic core equipped with hydrophilic side chains.[10]

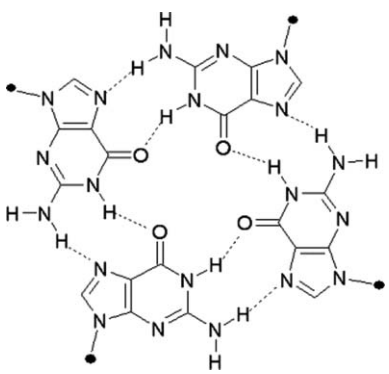

Fig. 1 Schematic representation of a G-tetrad, showing Hoogsteen hydrogen bonds.

In recent years, triazatruxene (Fig. 2) derivatives have been of great interest in supramolecular chemistry and in particular in organic electronics, with regard to their potential applications as light-emitting diodes,[11] battery and capacitors.[12] For their intrinsic photophysical and redox properties and their π-stacking capability,[13] these molecules can also behave as electroactive discotic liquid-crystals.[14] More recently, a triaza-fullerene was synthesized, using the triazatruxene core as a precursor.[15] Nevertheless, so far, triazatruxene derivatives have not been reported for important pharmaceutical applications and we thought that this kind of compound could represent a useful basis to develop new G-quadruplex ligands, provided suitable hydrophilic side chains were added to the triazatruxene moiety.

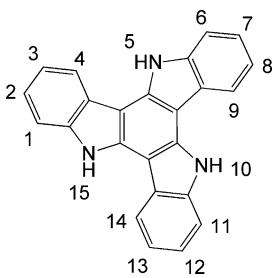

Fig. 2 Chemical structure of the triazatruxene core, showing its complete numbering.

Results and discussion

The triazatruxene core consists of a C3 symmetric cyclotrimer of indoles, which presents a wide aromatic surface with three useful points for the attachment of side chains, namely the three indolic NH at 5, 10 and 15 positions (Fig. 2). Previously, the synthesis of this core has been reported by reaction of indole with bromine,[16] leading to a mixture of brominated symmetric indole trimers, and subsequent dehalogenation.[17] In our experience, this way resulted in uncontrolled reactions with poor yields. So, we have considered another synthetic strategy, starting from N-substituted-2-indolones, which are reacted with

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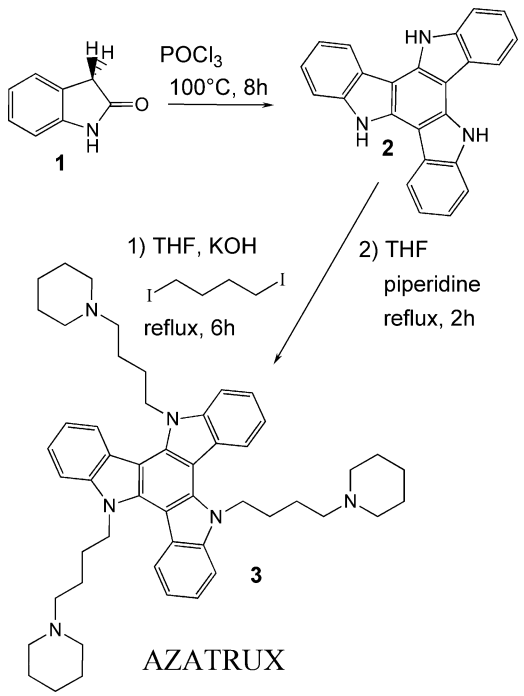
† Electronic supplementary information (ESI) available: Detailed synthetic procedures, molecular modelling simulations protocol, full characterization of the synthesized compounds, ^{1}H-NMR spectrum of AZATRUX as a hydrochloride in D_2O, full data of ESI-MS competition experiments and *in vitro* antitumor activities. See DOI: 10.1039/b904723a

phosphoryl chloride to give the corresponding tris-N-susbstituted-triazatruxenes.[18] Since the preparation of N-substituted precursors with hydrophilic chains can strongly limit the variability of the molecular features of possible substituents,[19] we have found more convenient the symmetric cyclotrimerization of 2-indolone **1** in $POCl_3$ at 100 °C,[20] leading to the unsubstituted azatruxene core **2** in 48% yield (Scheme 1). Attachment of the basic side chains consists of two steps: the N-alkylation in basic conditions with an excess of diiodobutane to give the corresponding tris-N-substituted derivative and subsequent substitution of the iodide at the end of the chain with an excess of piperidine to get the target compound **3**.‡ It is worth noting that the proposed pathway represents a versatile synthetic approach for the preparation of many compounds of this series with different side chains in terms of length and basicity, which are two critical parameters for modulating G-quadruplex ligands properties.[7]



Scheme 1 Synthetic scheme for the preparation of AZATRUX.

The new compound AZATRUX shows optimal molecular features for the interaction with the G-quadruplex, as confirmed by molecular modelling simulations:[8] the triazatruxene core stacks upon the terminal G-tetrad (Fig. 3a), while the hydrophilic side chains fit the DNA grooves (Fig. 3b). Since drug self-aggregation has been shown to be related to a lower telomerase inhibition and weaker interactions with the G-quadruplex with respect to unstacked molecules,[21] the self-aggregation in aqueous solution of G-quadruplex ligands must be carefully considered in the model of their interaction with G-quadruplex DNA.[7,8] For this reason, we have studied the self association of AZATRUX in MES-KCl buffer by UV-vis absorption spectroscopy (Fig. 4). The poor variability of the spectrum appearance when increasing temperature suggests that no significant self-association occurs, as confirmed by the clearly resolved aromatic region of the ^{1}H-NMR spectrum in D_2O (Fig. S1, ESI†).[22]

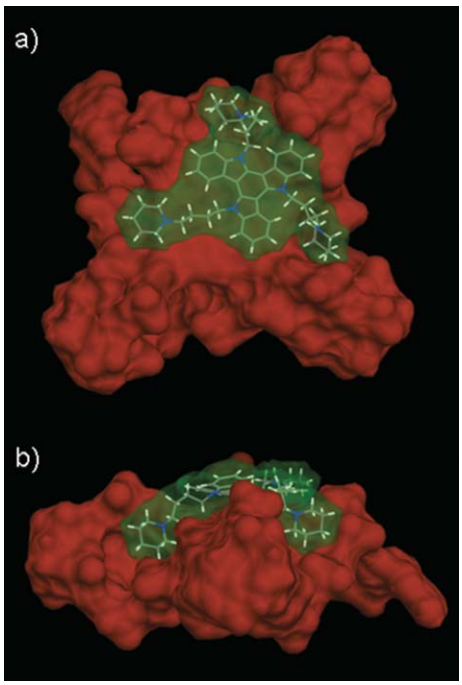


Fig. 3 Complex of AZATRUX (stick model with yellow transparent surface) with the human monomeric G-quadruplex DNA[25] (red surface), obtained by simulated annealing:[8] (a) top view, (b) lateral view.

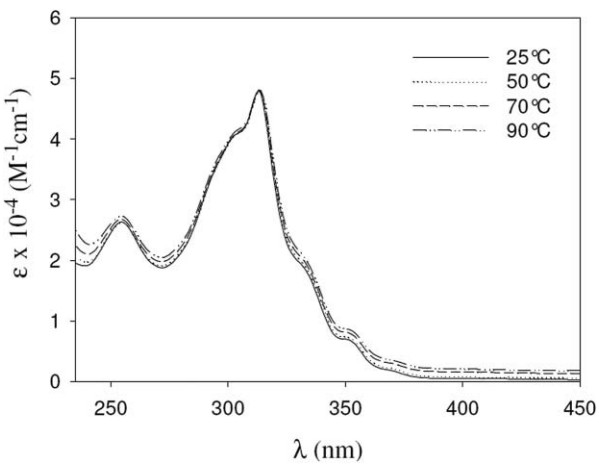


Fig. 4 UV-vis absorption spectra of AZATRUX in MES-KCl aqueous buffer at different temperatures.

The efficiency of AZATRUX in binding the G-quadruplex and its selectivity with respect to duplex DNA have been studied by electrospray ionization mass spectrometry (ESI-MS),[23] according to the protocol described in a previous paper.[24] For this study, we have chosen two oligonucleotides that can form different G-quadruplex structures: TG_4T (5′-TGGGGT-3′) which gives a tetrameric G-quadruplex and F21T (5′-GGGTTAGGGTTAGGGTTAGGG-3′) which is composed by human telomeric repeats and is able to fold in a monomeric G-quadruplex structure, characterized by X-ray crystallography in K^+ solution.[25] The formation of stable complexes between azatrux and the G-quadruplex forming oligonucleotides is clearly demonstrated by the presence in the mass spectra (at 1:1 drug/quadruplex ratio) of intense peaks corresponding to

Table 1 K_1 and K_2 values on a logarithmic scale for the complexes between AZATRUX and the indicated oligonucleotides as derived by ESI-MS experiments[24]

Oligo	Log K_1 [a]	Log K_2 [a]
$(TG_4T)_4$	6.3 ± 0.2	5.3 ± 0.2
F21T	5.5 ± 0.1	5.0 ± 0.1
DK66	4.1 ± 0.1	n.d.

[a] Standard deviations are reported over at least three independent experiments.

drug-quadruplex complexes (Fig. 5 and Fig. S2, ESI†). The evaluation of the binding constants obtained by the collected data (Table 1)[26] demonstrates that the studied molecule is a good G-quadruplex ligand able to form both 1:1 and 2:1 drug–DNA complexes. This agrees with the general model of a G-quadruplex–ligand interaction characterized by the presence of two binding sites on the external tetrad surfaces of the G-quadruplex structures (Fig. 3), even though other explanations are possible for the 2:1 stoichiometry, since the external tetrads of the quadruplex are not identical. The values of K_1 are above 10^5 for F21T and above 10^6 for $(TG_4T)_4$, while the order of magnitude for K_2 is 10^5 with both the oligonucleotides. Comparing K_1 and K_2 we can assume that no cooperativity is involved in the binding mechanism since K_2 values are always lower than those for K_1.[24]

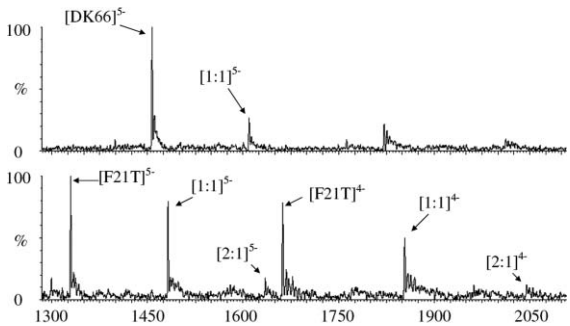


Fig. 5 ESI mass spectra of DK66 in the presence of AZATRUX at 1:4 molar ratio (top) and F21T in the presence of AZATRUX at 1:1 molar ratio (bottom). Arrows indicate the peaks corresponding to the oligos alone and to the relative 1:1 or 2:1 drug/DNA complexes, in different charge states.

In order to evaluate the selectivity of AZATRUX for quadruplex over duplex DNA we have studied preliminarily its affinity for a self complementary dodecamer: DK66 (5′-CGCGAATTCGCG-3′), one of the simplest duplex models widely used in the literature.[27] In this case, the spectra acquired at 1:1 ratios do not show any trace of the 1:1 or 2:1 complex peaks, suggesting a weaker interaction with respect to that revealed for G-quadruplexes. In order to detect an appreciable peak of the 1:1 drug/DNA complex, this molecule must be present in the sample at 3:1 and 4:1 ratios, but even at this concentration, there is no evidence of the peak relative to the 2:1 complex (Fig. 5). The order of magnitude for K_1 with DK66 is 10^4, about 25 times lower than that of K_1 with F21T and more than 150 times lower than K_1 with TG_4T (Table 1). These values suggest a good selectivity for quadruplex structures with respect to duplex DNA. Nevertheless, we have previously shown that a short duplex oligonucleotide is a really simplified model and physiologically nonrelevant interactions are possible with this kind of model.[24] In order to achieve a more reliable study of selectivity, we have performed competition experiments with the same technique, carried out in the simultaneous presence of G-quadruplex forming oligonucleotides and fragments of a double-stranded genomic DNA in 1:1 and 1:5 ratios, calculated on the basis of the phosphate group concentrations. Provided that calf thymus DNA (CT) cannot be detected in the used experimental conditions due to its high molecular weight, it is possible to report the percentage of bound quadruplex DNA[28] in the presence of different duplex concentrations (Fig. 6): any decrease of this percentage must be caused by the drug binding to genomic duplex DNA.[24]

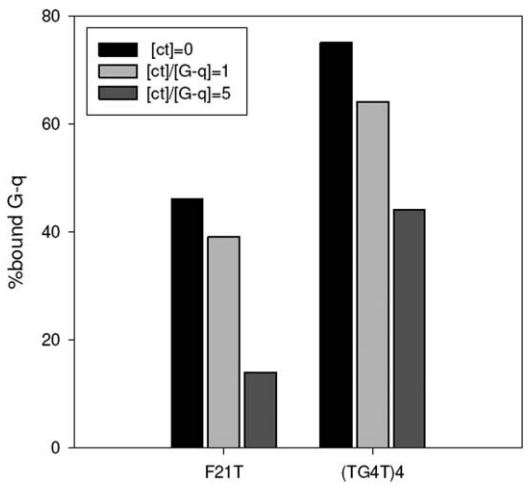


Fig. 6 Competition experiments on F21T and $(TG_4T)_4$ oligos. Histogram diagrams reporting the percentage of bound quadruplex DNA[24] (%bound G-q) for samples containing a fixed amount of both drug and G-quadruplex DNA (5 μM, 1:1 ratio) and different amounts of calf thymus DNA (CT), at the indicated duplex/quadruplex ratios (in phosphate ions). All values should be considered with an error estimated on at least three independent experiments of about ±5%.

The analysis of the measures performed on both oligonucleotides shows that the percentage value of quadruplex bound at a 1:1 duplex/quadruplex ratio has a poor decrease: from 75 to 64 with TG_4T and from 46 to 39 with F21T, which is more than 80% left with respect to the value in the absence of CT (Tables S1 and S2, ESI†). This result confirms the strong preference of this molecule for the quadruplex structure of DNA. At a 5:1 duplex/quadruplex ratio, AZATRUX is still able to bind 44% of the quadruplex DNA formed in the sample by TG_4T, which is about 2/3 of the quadruplex bound in the absence of duplex DNA, while in the case of F21T the percentage of quadruplex bound is remarkably reduced, probably due to a weaker affinity for this oligonucleotide with respect to TG_4T, even alone (Fig. S3, ESI†).

As an independent confirmation of this quadruplex/duplex selectivity, we have performed titrations of the human telomeric F21T oligonucleotide and the biologically relevant CT DNA with AZATRUX by UV spectroscopy (Fig. S4, ESI†). We noticed that the binding curve slope for quadruplex DNA is steeper than for duplex DNA and thus saturation is reached at a lower ratio in the first case. This different behaviour of AZATRUX towards the two DNA structures confirms a stronger interaction for telomeric

Table 2 Growth percent of the indicated cell lines after 48 hours of incubation with 10 μM AZATRUX solution, with respect to no-drug control, as reported by a preliminary study at the National Cancer Institute (NIH, Bethesda, MD, USA)[29]

Cell line	Tumour	Growth percent (%) [a]
RPMI-8226	leukemia	–49.4
K-562	leukemia	–81.8
A549/ATCC	lung cancer	–49.7
HOP-62	lung cancer	–91.4
HCT-15	colon cancer	–68.4
HCT-116	colon cancer	–97.1
U251	CNS cancer	–68.4
SF-539	CNS cancer	–98.7
UACC-257	melanoma	–56.5
SK-MEL-5	melanoma	–96.6
IGROV1	ovarian cancer	–31.4
SK-OV-3	ovarian cancer	–98.8
RXF 393	renal cancer	–54.5
A498	renal cancer	–95.2
PC-3	prostate cancer	–93.2
DU-145	prostate cancer	–95.2
HS 578T	breast cancer	–48.0
MDA-MB-468	breast cancer	–93.0

[a] Only the lowest and the highest values for each tumour type are reported, the complete data over 60 cell lines have been included in the ESI†.

G-quadruplex with respect to genomic duplex DNA, also in these conditions (50 mM KCl solution) more similar to physiological conditions than those used in ESI-MS experiments.

A preliminary study at the National Cancer Institute (USA) on the effects of AZATRUX on the growth of 60 different tumour cell lines[29] suggests a good potential anticancer activity in vitro (Table 2). Growth percents after 48 hours of incubation with 10 μM AZATRUX solution range from –31 to –99%, with respect to no-drug control, suggesting a potentially selective growth inhibition for different tumour types. Following these results, NCI has decided to perform further multi-dose studies.

Conclusion

From the reported data, the triazatruxene derivative AZATRUX results in a potent and selective G-quadruplex ligand, which surely deserves further investigation on its biological activity. In fact, due to its high water solubility and DNA binding properties, this new compound is very promising in terms of biological and possible pharmacological effects, not only at telomeres but also with respect to regulation of several oncogenes.[10] The results of a preliminary study on the effects of this molecule on the growth of several different tumour cell lines suggest an interesting and possibly selective inhibitory action, although obviously at the moment it is not possible to correlate this finding to quadruplex binding. Moreover, the versatility of the synthetic pathway has been applied in our lab to synthesize other compounds of this series, with side chains of different length and basicity, which will soon be reported in a complete comparative study.

Acknowledgements

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Notes and references

‡ Details on materials, instrumentation and synthetic procedures have been provided in the Electronic Supplementary Information†, together with the full characterization of all the intermediate compounds.

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