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Interactions of cryptolepine and neocryptolepine with unusual DNA structures

Lionel Guittat [a,1] Patrizia Alberti [a,1] Frédéric Rosu [b,1] Sabine Van Miert[c], Emilie Thetiot [a], Luc Pieters [c], Valérie Gabelica [d], Edwin De Pauw [d], Alexandre Ottaviani [a], Jean-François Riou [e], Jean-Louis Mergny [a]

[a] *Laboratoire de Biophysique, Muséum National d'Histoire Naturelle, INSERM UR 565, CNRS UMR 8646, 43, rue Cuvier, 75231 Paris cedex 5, France*

[b] *Biospectroscopy Laboratory, Chemistry Institute (B6c), University of Liege, Allée de la chimie, 17, 4000 Liege (Sart-Tilman), Belgium*

[c] *Department of Pharmaceutical Sciences, University of Antwerp, Universiteitsplein 1, 2610 Antwerp, Belgium*

[d] *Mass Spectrometry Laboratory, Department of Chemistry, University of Liege, Belgium*

[e] *Laboratoire de Physiologie Humaine, UFR de Pharmacie, IFR 53, Université de Reims Champagne-Ardenne, 51, rue Cognacq-Jay 51096 Reims cedex, France*

[1] These authors contributed equally to this work.

Abstract

Cryptolepine, the main alkaloid present in the roots of *Cryptolepis sanguinolenta,* presents a large spectrum of biological properties. It has been reported to behave like a DNA intercalator with a preference for GC-rich sequences. In this study, dialysis competition assay and mass spectrometry experiments were used to determine the affinity of cryptolepine and neocryptolepine for DNA structures among duplexes, triplexes, quadruplexes and single strands. Our data confirm that cryptolepine and neocryptolepine prefer GC over AT-rich duplex sequences, but also recognize triplex and quadruplex structures. These compounds are weak telomerase inhibitors and exhibit a significant preference for triplexes over quadruplexes or duplexes.

Keywords*:* Cryptolepine; Triplex; Quadruplex; Telomere; Telomerase

1. Introduction

Cryptolepine (5-methyl indolo[2,3b]-quinoline, Fig. 1A, left), a natural alkaloid, was first isolated from the roots of *Cryptolepis triangularis* [1-3]. This compound is the main alkaloid present in the roots of *Cryptolepis sanguinolenta,* a plant traditionally used in Central and West Africa for the treatment of rheumatism, urinary and respiratory infections. Extracts of the roots are used in decoction to treat fevers due to malaria and stomach disorders [4]. Cryptolepine presents a large spectrum of biological properties, including hypotensive and antipyretic, antimuscarinic, antibacterial and antiinflammatory effects [5]. It also possesses potent activity in vitro against *Plasmodium falciparum,* which is one of the main parasite species responsible for malaria. The mechanism of action of this antimalarial product remains unclear; at least two independent effects may together lead to a potent activity. First, it behaves like a DNA intercalator with a preference for GC-rich sequences [5]. Second, it may act like chloroquin by inhibiting the detoxification of haeme in red blood cells [6]. It has been proposed that crytolepine exerts its cytotoxic action via the inhibition of DNA synthesis and stabilization of topoisomerase II-DNA covalent complexes [5]. Neocryptolepine differs from the parent isomer only in terms of the orientation of the indole unit with respect to the quinoline moiety (see Fig. 1 A, right). It also intercalates into GC-rich sequences and interferes with the catalytic activity of human topoisomerase II, although less efficiently than cryptolepine [7]. Cryptolepine and neocryptolepine have cytotoxic effects and induce apoptosis in HL60 leukaemia cells [8]. Recently, the structure of a cryptolepine-DNA complex was elucidated by X-ray crystallography. Lisgarten et al. [9] demonstrated that the drug interacts with the CC sites of the $d(CCTAGG)_2$ oligonucleotide; this is the first intercalator to bind non-alterning (pyrimidine-pyrimidine) DNA sequence [9]·

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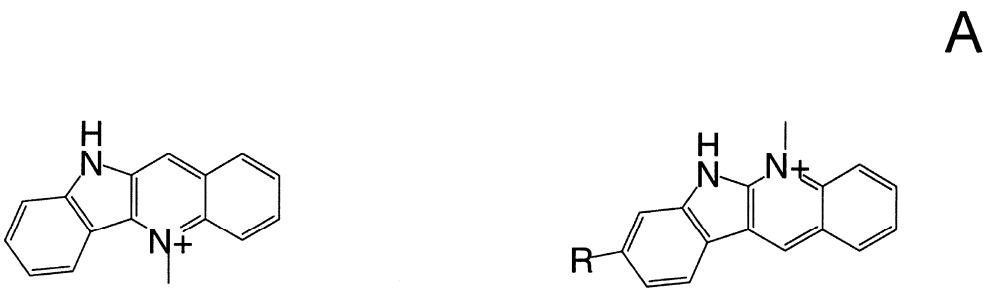
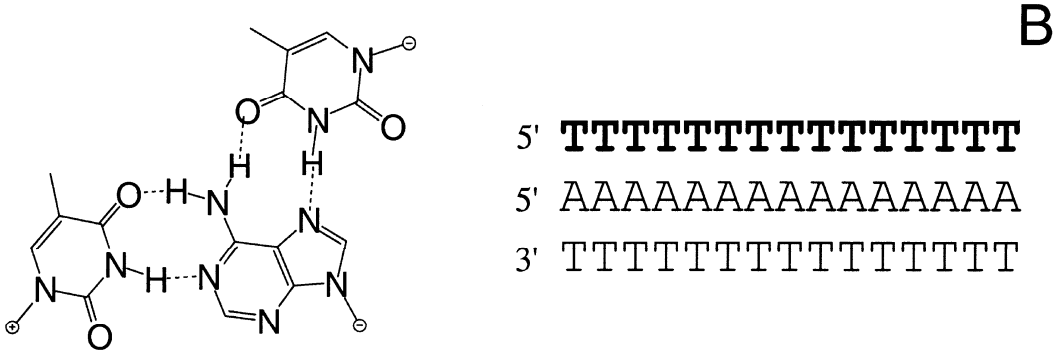
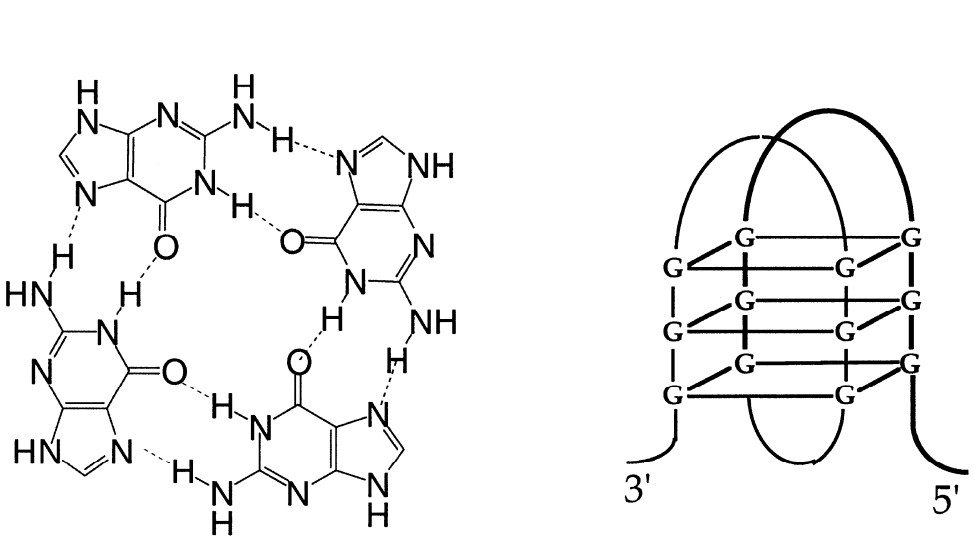
Fig. 1. *(A) Formulae of cryptolepine (5-methyl indolo[2,3b]-quinoline, left), neocryptolepine (R = H) and derivatives (R = Cl, Br and OCH_3) (right). (B) T.A*T triplet (left) and a schematic diagram of an oligo(dA)/2oligo(dT) triple-helix, showing the relative orientation of the strands. (C) G-quartet (left) and a schematic diagram of intramolecular antiparallel G-quadruplexes involving three quartets.*







The binding mode of these derivatives to double-stranded DNA has been extensively studied. However, besides duplexes, DNA is prone to structural polymorphism and a number of alternative DNA structures have been described to date. DNA conformation may differ from a regular double-helix and may involve the association of more than two strands, leading to the formation of triplexes and quadru-plexes. Alternative DNA structures offer significant differences in terms of electrostatics, shape and rigidity compared to single- or double-stranded DNA. Therefore, specific recognition of unusual DNA structures, such as triplexes and quadruplexes by small molecules should be possible.

Short oligonucleotides can bind to the major groove of DNA duplexes to form a triple helix (Fig. 1B) [10,11]. Triplex-forming oligonucleotides (TFOs) can interfere with the binding of proteins [12] and affect the transcription of a specific gene [13]. At least three classes of triplexes exist which differ in sequence composition and relative orientations of the phosphodiester backbone of the third strand [14]. Nevertheless, triplex stability under physiological conditions is relatively low and this limitation led to the design of small molecules that interact with the triplex and stabilize it [15] or of chemical modifications that increase the affinity of the third strand for its target [16].

G-quadruplexes are a family of secondary DNA structures formed in the presence of monovalent cations that consist of four-stranded structures stabilized by G-quartets (Fig. 1C). There is a renewed interest in G-quadruplex structures due to their putative biological regulatory function [17,18]. The 3' terminal region of the G-rich strand of human telomeres is single-stranded and may adopt a G-quadruplex conformation [19]. This structure has been shown to directly inhibit telom-erase elongation in vitro [20]. Telomeres protect chromosomal ends from fusion events and provide a means for complete replication of the chromosome. Telomerase is expressed in tumour cells, but not in most somatic cells. Therefore, a drug that stabilizes quadruplexes could interfere with telomere elongation and replication of cancer cells. The recent discovery of positively charged aromatic compounds (for a review [21]) that interact with G-quartet led us to test the binding of cryptolepine and neocryptolepine analogues to G-quadruplexes. A cytidine-rich oligomer forms a radically different quadruplex in which two parallel C-strands associate in a head-to-tail orientation with their $C\bullet C^+$ pairs face-to-face, intercalated in a so called i-motif or C-quadruplex [22,23].

The aim of the present work is to study the binding and/or stabilization of cryptolepine and its regio selective isomer neocryptolepine to a panel of different DNA structures. Dialysis competition assay and mass spectrometric experiments were used to determine the affinity of cryptolepine for different DNA structures (duplexes, triplexes, quadruplexes and single strands). Our data confirm that cryptolepine prefers GC over AT-rich duplex sequences, but also recognizes triplex and quadruplex structures. In order to strengthen these results, we performed UV and fluorescence melting experiments. Finally, we studied the effect of these compounds on telomerase activity by a telomerase repeat amplification protocol (TRAP) assay.

2. Materials and methods

2.1. Compounds

Cryptolepine was isolated from *Cryptolepis sanguino-lenta* [24]. Neocryptolepine (as well as 2-C1-, 2-Br- and 2 methoxyneocryptolepine) was obtained by organic synthesis as previously described [25]. Powders were resuspended at high concentrations (mM) in DMSO (Sigma) and then diluted in bidistilled water for further experiments. The compounds were evaluated as their hydrochloride salt.

2.2. Nucleic acids

Oligodeoxynucleotide probes were synthesized by Euro-gentec (Belgium) on the 1 μmol scale. Purity was checked by gel electrophoresis. All the concentrations were expressed in strand molarity using a nearest-neighbour approximation for the absorption coefficients of the unfolded species. All polynucleotides were ordered from Amersham-Pharmacia. F21MB is a doubly labelled 21 base-long oligomer that mimics the human telomeric guanine-rich strand with fluorescein at the 5' endandDABCYL at the 3' end, respectively. It was synthesized and purified by Eurogentec (Belgium).

2.3. Dialysis experiments

The initial dialysis protocol was defined by Ren and Chaires [26]. We have adapted this test to accommodate a different set of nucleic acid structures [27]. Briefly, a buffer consisting of 15 mM sodium cacodylate (pH 6.5), 10 mM $MgCl_2$, and 185 mM NaCl was used for all the experiments. All structures used in these experiments

were stable at room temperature under the chosen experimental conditions [28]. In particular, the presence of 185 mM sodium chloride promotes quadruplex formation: the melting temperature of the quadruplexes was found to be 62 °C for the human telomeric motif and > 90 °C for the parallel quadruplex [28]. 400 ml of the dialysate solution containing 1 µM ligand were used for each competition dialysis assay. A volume of 200 µl at 75 µM monomelic unit (nucleotide, base pair, base triplet or quartet) of each of the nucleic acids samples was pipetted into a separate Dialyzer unit (Pierce). All 19 dialysis units were then placed in the beaker containing the dialysate solution. The beaker was covered with Parafilm and wrapped in foil, and its contents were allowed to equilibrate with continuous stirring at room temperature (20-22 °C) overnight. At the end of the equilibration period, DNA samples were carefully removed to microfuge tubes, and treated with 1% SDS. The ligand concentration in each sample was determined by fluorescence spectroscopy (excitation at 320 nm for cryptolepine and neocryptolepine). The amount of dye in each dialysis unit is directly proportional to the fluorescence emission intensity signal of the sample as the nucleic acid-compound complex is dissociated by SDS. This quantification procedure has been validated in a number of independent studies by several groups, including ours [26,28-31].

A panel of 19 different nucleic acid structures were used (Table 1). They have been described in detail elsewhere [27,28]. The TC, GA and GT triplexes result from the association of two strands of different lengths (13 and 30 nucleotides) [14]. The GA duplex results from the self-association of a $(GA)_{12}$ oligonucleotide. The parallel-stranded psD duplex results from the association of two AT strands. The 24CTG mimics eight repeats of the trinucleotide unit $(CTG)_8$. ds26 is a duplex formed with a self-complementary oligonucleotide. 22CT is an oligonucleotide that mimics the cytosine-rich strand of human telomeres [32], whereas 22AG is an oligonucleotide that mimics the guanine-rich strand of human telomeres. 24G20 may form an intermolecular G-quadruplex, whereas poly(dC) may form an intermolecular i-motif. Single strands DNA are represented by poly(dT) and poly(dA). Poly (rU) and poly(rA) represent single-stranded RNA.

Table 1. *List of the oligonucleotides used in this study*

Triplex 14C3	**5'-TTCTTCTTTTTTCT-3'** 5'-GATACGAGTTAAGAAGAAAAAAGATTGAGCTGATAG-3'
(T,C) triplex	3'-CTATCAGCTATTCTTCTTTTTTCTAACTCGACTATC-5'
	TT-CTTTCTCTCCTCC-3 5'-GAAAGAGAGGAGG-3' CC-CTTTCTCTCCTCC-5'
(G,A) triplex	TT-GGAGGAGAGAAAG-3'
	CC-GGAGGAGAGAAAG-5' **5'-CCTCCTCTCTTTC-3'**
(G,T) triplex	TT-GGTGGTGTGTTTG-3'
	CC-GGAGGAGAGAAAG-5' **5'-CCTCCTCTCTTTC-3'**
24GA (parallel duplex)	5'-GAGAGAGAGAGAGAGAGAGAGAGA-3'
PSD (parallel duplex)	5'-AAAAAAAAAATAATTTTAAATATT-3'
	5.TTTTTTTTTTATTAAAATTTATAA-3'
24CTG (trinucleotide repeat)	3'-GTCGTCGTCGTCGTCGTCGTCGTC-5'
DS26 (intramolecular duplex)	3'-GTTAGCCTAGCTTAAGCTAGGCTAAC-5'
22CT (intramolecular i-motif)	5'-CCCTAACCCTAACCCTAACCCT-3'
22AG (intramolecular G-quartet)	5'-AGGGTTAGGGTTAGGGTTAGGG-3'
24G20 (intermolecular G-quartet)	5'-TTGGGGGGGGGGGGGGGGGGGTT-3'

2.4. Mass spectrometry

Single-stranded oligodeoxyribonucleotides d(CG-TAAATTTACG) (MW = 3644.45), d(CGCGAATTCGCG) (MW = 3646.44 Da), d(CGCGGGCCCGCG) (MW = 3678.40), d(TGGGGT) (M = 1863.26), d(GGGG-TTTTGGGG) (MW = 3788.50), d((GGGTTA)$_3$GGG) (MW = 6653.35), d(CCTTTTCTCTTTCC) (MW = 4106.71), d(G-GAAAGAGAAAAGG) (MW = 4418.96), d(CCTTT-CTCTTTTCC) (MW = 4106.71) were purchased from Euro-gentec (Belgium) and used without further purification. Duplex and quadruplex solutions were prepared, respectively, in 100 and 150 mM NH_4OAc, pH 7.0. The triple helix solution involves the association of three independent strands (5'dCCTTTTCTCTTTCC, 5'dGGAAAGAGAAAAGG and 5'dCCTTTCTCTTTTCC)12 and was prepared in 150 mM NH4OAc acidified with acetic acid (pH 5.5). Oligonucleotide solutions were heated to 85 °C for 5 min and then slowly cooled down to 20 °C to form the desired duplex, quadruplex or triplex structures. The extinction coefficients for the unfolded single strands were provided by the supplier. Circular dichroism and UV melting experiments were used to characterize the solution conformation of the oligonucleotide in ammonium acetate [33]. Experiments on the duplex structures were performed on an LCQ mass spectrometer (Finnigan, Can Jose, CA, USA) as described previously [34]. Similar results are obtained on the Q-TOF instrument. Experiments on the quadruplexes and triplexes were performed on a Q-TOF Ultima Global apparatus (Micromass, Manchester, UK) operated in the negative ion mode. The experimental conditions were optimized for the Z-spray source to avoid denaturation of the duplex, triplex or quadruplex species. The source parameters are slightly different from those used on the Q-TOF2 instrument used previously [33] : a source block temperature of 70 °C and a cone voltage of 75 V were used. An RF Lens 1 energy of 12 V (quadruplexes) and 20 V for triplexes and a source pressure of 2.8 mBar were necessary to observe fully desolvated complex species. Spectra of mixtures of DNA (C = 5 µM) + (neo)cryptolepine (C = 10 µM) were recorded. Methanol (15%) was added to the samples just before injection in order to obtain a stable electrospray signal. The rate of sample infusion into the mass spectrometers was 4 µl/min. As previously discussed [34], the relative intensities of the free and bound DNA in the mass spectra are assumed to be proportional to the relative abundances of these species in solution. As the starting concentrations are known, the concentrations of all individual species at equilibrium (free DNA, 1:1 complex, 2:1 complex and, by difference, the free drug) can be determined from the relative intensities of the free DNA and the complexes, and the equilibrium binding constants can be determined. The affinity of the ligand for the DNA structure can also be characterized by the concentration of bound ligand per DNA molecule, or per binding unit. The concentration of bound ligand per DNA molecule is obtained with the following Eq. (1):

$$[\text{Bound Ligand}] = C_0 \cdot (I_{(1:1)} + 2.I_{(2:1)} + 3.I_{(3:1)})/(I_{DNA} + I_{(1:1)} + I_{(2:1)} + I_{(3:1)}) \quad (1)$$

where C_0 is the starting DNA concentration, I_{DNA} is the relative intensity of the DNA, and $I_{(n:1)}$'s are relative intensities of the complexes *(n* drug molecules bound to 1 DNA target). The relative intensities were obtained from a sum of 60 spectra. The amount of bound ligand expressed in molecular binding unit (base pairs, base triplet or quartet in the DNA target) is determined by dividing the total amount of bound ligand by the number of monomelic units.

2.5. FRET studies

FRET can be used to probe the secondary structure of oligodeoxynucleotides mimicking repeats of the guanine-rich strand of vertebrate telomeres, provided that a fluorescein molecule (donor) and a DABCYL derivative (quencher) are attached to the 5' and 3' ends of the oligonucleotide, respectively. FRET measurements with the F21MB oligonucleotide were performed on a Spex Fluorolog DM IB instrument, using a bandwidth of 1.8 nm and 0.2 x 1 cm quartz cuvettes, containing 600 µl of solution in a 0.1 M lithium chloride, 10 mM sodium pH 7.2 cacodylate buffer [35]. Other buffers have been tested to demonstrate that a weak stabilization is still observed under different experimental conditions. The temperature of the circulating water bath was recorded at regular time intervals. The melting of the G-quadruplex was monitored in the presence and or in the absence of the dye by measuring the fluorescence of the donor. A concentration of 1-3 µM of cryptolepine derivatives was chosen.

2.6. UV-melting studies

All the thermal denaturation profiles were obtained with a Kontron Uvikon 940 spectrophotometer as described [36]. The temperature of the thermal bath was increased or decreased at a rate of 0.2 °C/min, thus allowing complete thermal equilibrium of the cells. The experiments with the standard triplex 14C3 were performed in a

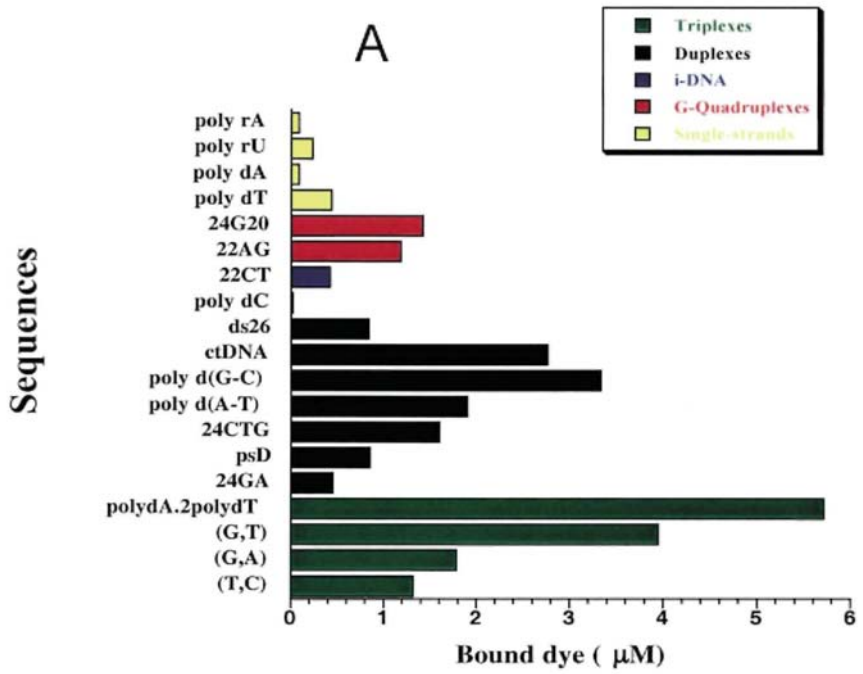
10 mM sodium cacodylate (pH 6.2) with 100 mM NaCl and the following oligonucleotide concentrations: 1.5 µM for 36R and 36Y, 1.8 µM forthe third strand 14C3 and 6 µM cryptolepine [15].

UV melting studies on dialysis sequences were carried out in the dialysis buffer (15 mM sodium cacodylate, 10 mM $MgCl_2$ and 185 mM NaCl, pH 6.5) at 1.5 µM strand concentrations (equivalent to 19.5 µM in base triplets) and 6 µM cryptolepine. The concentration in base triplets was 19.5 µM for poly(dA)/2poly(dT) with 6 µM of cryptolepine. The poly(dA)/2poly(dT) was also tested in different buffer conditions (10 mM sodium cacodylate and 100 mM NaCl (pH 6.2) and 5 mM $MgCl_2$) and cryptolepine concentration (3 µM). For all the experiments, a 540 nm wavelength control was registered and the denaturation of the complex was followed at 260 and 295 nm (for DNA) and 366 and 379 nm (for cryptolepine).

2.7. TRAP assay

The TRAP reaction was performed as previously described [37] in a 20 mM Tris HC1 pH 8.3 buffer containing 63 mM KC1, 1.5 mM $MgCl_2$, 1 mM EGTA, 0.005% Tween 20, 0.1 mg/ml BSA, 50 µM dTTP, dGTP and dATP, 10 µM dCTP, 0.1 µg of the TS (5'-AATCCGTCGAGCAGAGTT-3 ') and CX primers, 5 ng of the NT primer, (5'-ATCGCTTCTCGGCCTTTT-3') 0.05 ng of the TSNT primer (5'-ATTCCGTCGAGCAGAGTTAAAAGGCCG-AGAAGCGAT-3'), 2 units of Taq polymerase, 2 µCi/tube of $dCTP^{32}$ and 200 ng of A549 CHAPS extracts. After telom-erase elongation for 15 min at 30 °C, 30 cycles of PCR were performed (94 °C 30 s, 50 °C 30 s and 72 °C for 90 s). Telomerase extension products were then analysed on a non-denaturing 12% polyacrylamide 1X Tris Borate EDTA (TBE) vertical gel.

Fig. 2. *Equilibrium dialysis. All the measurements were performed in a 185 mM NaCl, 10mM $MgCl_2$, 15 mM Na-cacodylate buffer (pH 6.5). The nucleic acid names are given on the left and all the structures are described in Section 2 and in Table 1. (A) Cryptolepine. (B) Neocryptolepine.*



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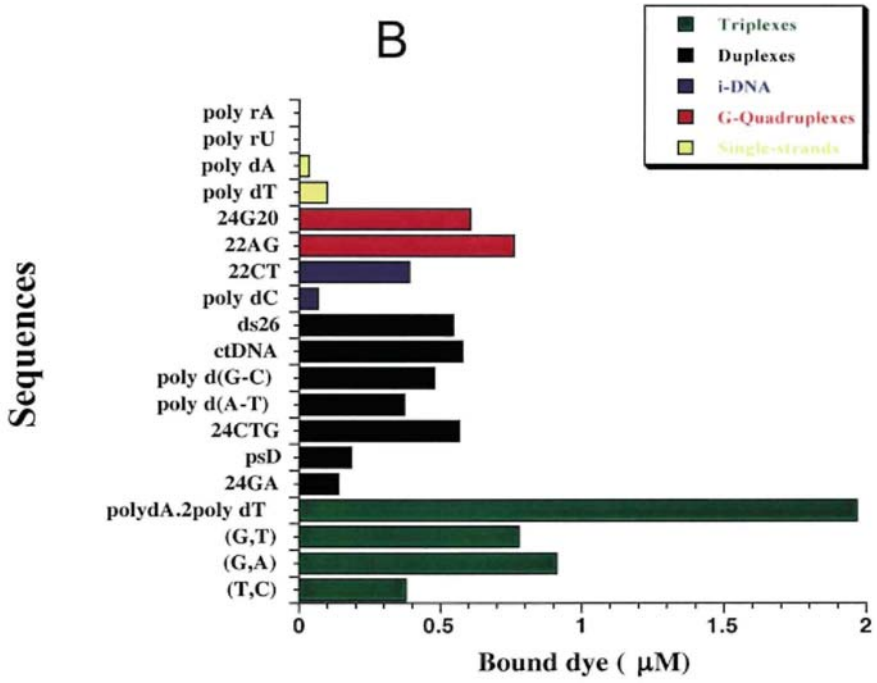

3. Results

3.1. Equilibrium dialysis

To evaluate the selectivity of cryptolepine and neocryptol-epine for different DNA structures, we performed a competitive dialysis experiment using 19 nucleic acid structures (described in Section 2) against a common (neo)cryptolepine solution. More products accumulate in the dialysis tube containing the structural form with the highest ligand binding affinity. It is possible to correlate the amount of the bound dye to a given structure with the affinity of the dye for that sample. As shown in Fig. 2A, cryptolepine interacts preferentially with duplex and triplex structures, whereas it shows a weak affinity for i-motif, single strands and a moderate affinity for G-quadruplexes. For cryptolepine (Fig. 2A) and neocryptolepine (Fig. 2B), poly(dA)/2poly(dT) appears to be the preferred structure. A comparison of the binding affinity of cryptolepine for different duplexes indicates a preference for poly d(G-C) and ct-DNA, whereas pSD, ds26 and 24CTG show much weaker binding. In agreement with a recent report, cryptolepine interacts preferentially with GC than AT sequences [9]. Dialysis equilibrium experiments also present evident differences in DNA affinity between neocryptolepine and cryptolepine, confirming previous results [7]. The binding profile of the two compounds relative to higher-order DNA structures was quite unexpected and prompted us to confirm this interaction using different techniques.

3.2. Mass spectrometry

The ability of mass spectrometry to investigate drug-DNA interactions has been reviewed recently [38,39]. The binding stoichiometry, the relative binding affinities and the binding constants for DNA double helices of various sequences may be determined [34] and DNA complexes with intercalators and minor groove binders have been studied [40,41]. DNA triplex and quadruplex structures may also be investigated by electrospray mass spectrometry [33]. The affinity of cryptolepine and the parent isomer neocryptolepine for various DNA forms was therefore analysed by electrospray mass spectrometry (ESI-MS). In the present study, three 12 base pair duplexes with different GC content, three quadruplexes $d(G_4T_4G_4)_2$, $d(TG_4T)_4$ and the human telomeric sequence $d[(G_3T_2A)G_3]$ (reminiscent of tetraplexes 24G20 and 22AG used in the equilibrium dialysis assays) and one triple helical DNA structure (referred to as triplex) were studied. Fig. 3 shows the MS spectra obtained with the triplex (see Section 2 for details), the $(TG_4T)_4$ quadruplex and the Dickerson dodecamer duplex $d(CGCGA_2T_2CGCG)_2$, with cryptolepine. A mixture of 1:1 and 2:1 drug-DNA complexes were detected with all

the sequences (also some 3:1 complexes with the triplex). The largest amount of complex is observed with the triplex, followed by the duplex. The amount of drug bound per quadruplex is lower than that for the other two structures. However, in order to allow the comparison with dialysis experiments, the amount of drug bound was expressed in terms of monomelic unit: the amount of bound drug calculated with Eq. (1) is divided by 14,12,and 4 for the triplex, duplex, and quadruplex, respectively. Fig. 4 summarizes these results for cryptolepine and neocryptolepine. The latter has a much lower affinity than the former for all DNA structures, but shows the largest discrimination between the triplex and the other DNA structures. Both the drugs interact preferentially with a duplex containing a high GC content as expected for these intercalators [7]. The ESI-MS results agree well with the equilibrium dialysis results and these two independent methods lead to an identical conclusion: a preferential binding of cryptolepine to triple helical DNA. To our knowledge, this is the first report of a drug-triplex interaction detected and quantified by electrospray mass spectrometry. We then wanted to confirm this interaction using spectroscopic techniques.

3.3. Triplex binding

Using UV-absorbance melting studies on the 14C3 system, we observe a triplex stabilization of 3 and 5 °C at 6 and 15 μM cryptolepine, respectively, in a 10 mM sodium ca-codylate and 100 mM NaCl pH 6.2 buffer. Under identical conditions, a relatively weak stabilization of a 21 base pair long telomeric duplex (ΔT_m = 3 °C) was observed at 8 μM (data not shown).

We then performed experiments on triplex sequences used in the dialysis experiments with the same buffer (15 mM sodium cacodylate, 10 mM $MgCl_2$, and 185 mM NaCl, (pH 6.5)). For the (T,C) (G,A) and (G,T) triplexes, the denatur-ation profiles did not significantly change in the presence of 6 μM cryptolepine. With regard to the poly(dA)/2 poly(dT) triplex, we observed two transitions without cryptolepine with a T_m of 65 and 75 °C, corresponding to the triplex and duplex dissociation, respectively. In the presence of 3 μM cryptolepine, only one transition is observed with a T_m of 75 °C (not shown). We performed this experiment in different buffer conditions (10 mM sodium cacodylate, 100 mM NaCl (pH 6.2) and 5 mM $MgCl_2$) and cryptolepine concentration (3 μM). Again, we observed two transitions without cryptolepine (T_m = 68 and 75 °C) and only one with the dye (T_m = 75 °C) (Fig. 5). Two hypotheses arise: either cryptolepine completely prevents triplex formation or stabilizes it (i.e. the first transition is merged with duplex melting). Two reasons favour the second hypothesis. First, the spectra of cryptolepine with poly(dT) and the duplex poly(dA)/poly(dT) show no significant differences at high and low temperatures, whereas we observed a change with the triplex revealing an interaction between the dye and the DNA triplex at low temperature (Fig. 6). Second, the sum of the amplitudes of the two transitions corresponds to 0.15 OD without cryptolepine (0.1 for duplex and 0.05 for triplex). The amplitude of the duplex transition with cryptolepine is 0.1 OD, whereas the triplex poly(dA)/2poly(dT) transition leads to a hyper-chromism of 0.15. If cryptolepine prevents triplex formation, no difference in amplitudes should be observed. Therefore, we can conclude that cryptolepine significantly stabilizes (ΔT_m = 10 °C) the poly(dA)/2poly(dT) triplex. An absor-bance titration of cryptolepine by the poly(dA)/2poly(dT) triplex was then performed in a 100 mM NaCl, 5 mM $MgCl_2$, 10 mM cacodylate (pH 6.2) buffer. The interaction between cryptolepine and the poly (dA)/2poly (dT) triplex is evidenced by a modification of the absorbance spectrum of the dye. A slight red-shift in the absorbance peak at 368 nm is associated with a significant decrease in the extinction coefficient (data not shown).

3.4. Quadruplex binding

Dialysis and mass spectrometry experiments suggest that cryptolepine interacts with quadruplexes. To confirm this observation, we performed a UV-visible titration of cryptolepine (10 μM) with increasing amounts of 22 AG (0-12 μM). This oligonucleotide mimics the repeats of the human telomeric motif, and may adopt an intramolecular quadruplex structure [42,43]. This titration was performed in the presence of 0.1 M KC1, i.e. under conditions that strongly favour quadruplex formation (Fig. 7).

The progressive modification of the absorbance spectrum of cryptolepine demonstrates an interaction with the preformed G-quadruplex. A slight red-shift in the absorbance peak at 368 nm is associated with a significant decrease in the extinction coefficient (=50%). A near isobestic point is obtained at 375 nm. However, no isobestic point is observed at around 400 nm, demonstrating that the binding process is complex and involves more than one binding mode. The absorbance values as a function of 22AG concentrations are shown in Fig. 7B. The shape of the titration curve suggests a relatively modest binding affinity (in the 10^5 M^{-1} range). The presence of several binding modes with different spectral properties (in agreement with the 1:1 and 2:1 complexes evidenced by MS) prevents a more precise analysis of the data.

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We monitored by FRET the melting temperature of a G-quadruplex (5' GGGTTAGGGTTAGGGTTAGGG-3' representing the human telomeric motif) in the presence of various concentrations of cryptolepine. The melting temperature of a G4-forming fluorescent oligonucleotide may be recorded in the presence of various concentrations of the ligand [35,44]. At 1 µM cryptolepine, there is no significant stabilization of the G-quadruplex, but a weak stabilization (ΔT_m = 3 °C) is observed at 3 µM (data not shown). In order to confirm these results, fluorescence melting assays with 1-3 µM cryptolepine were also performed under different ionic conditions: the equilibrium dialysis assay buffer (15 mM sodium cacodylate, 10 mM $MgCl_2$ and 185 mM NaCl, pH 6.5), the mass spectrometry buffer (0.1 M ammonium acetate) strongly favour quadruplex formation (0.1 M KC1). In all the cases, the stabilization was found to be 0-1.5 °C at 1 µM cryptolepine and between 1 and 4 °C at 3 µM dye concentration (data not shown). These results are in agreement with the equilibrium dialysis and absorbance measurement, which indicates that the interaction of cryptolepine with the quadruplex-forming-oligonucleotides is relatively weak. No significant stabilization is observed with neocryp-tolepine derivatives even at 3 µM.

Fig. 3. *Mass spectrometry. ESI-MS spectra of mixtures of 10 µM cryptolepine with (**A**) the triplex d(CCTTTTCTCTTTCC). d(GGAAAGAGAAAAGG)*d(CCTTTCTCTTTTCC). (**B**)the parallel quadraplex $d(TG_4T)_4$. (**C**) the dodecamer duplex $d(CGCGA_2T_2CGCG)_2$. Each structure was tested at a concentration of 5 µM. Spectra were recorded using the Q-Tof ultima Global.*

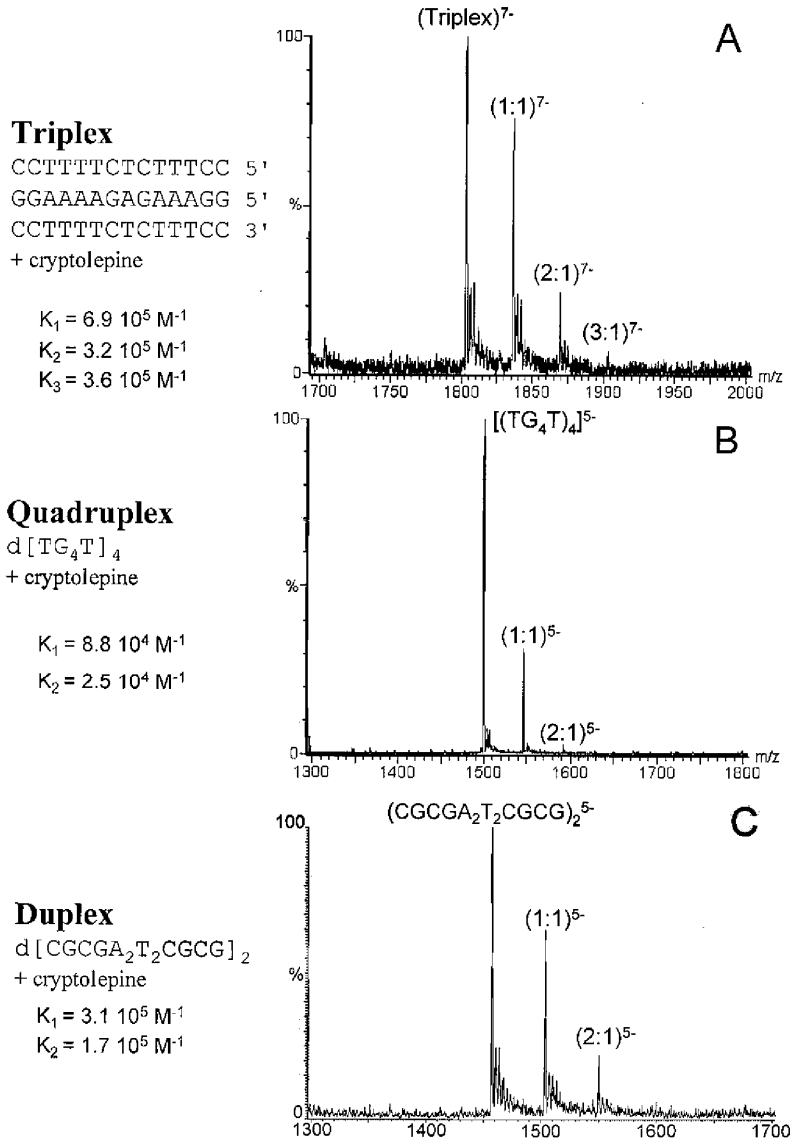
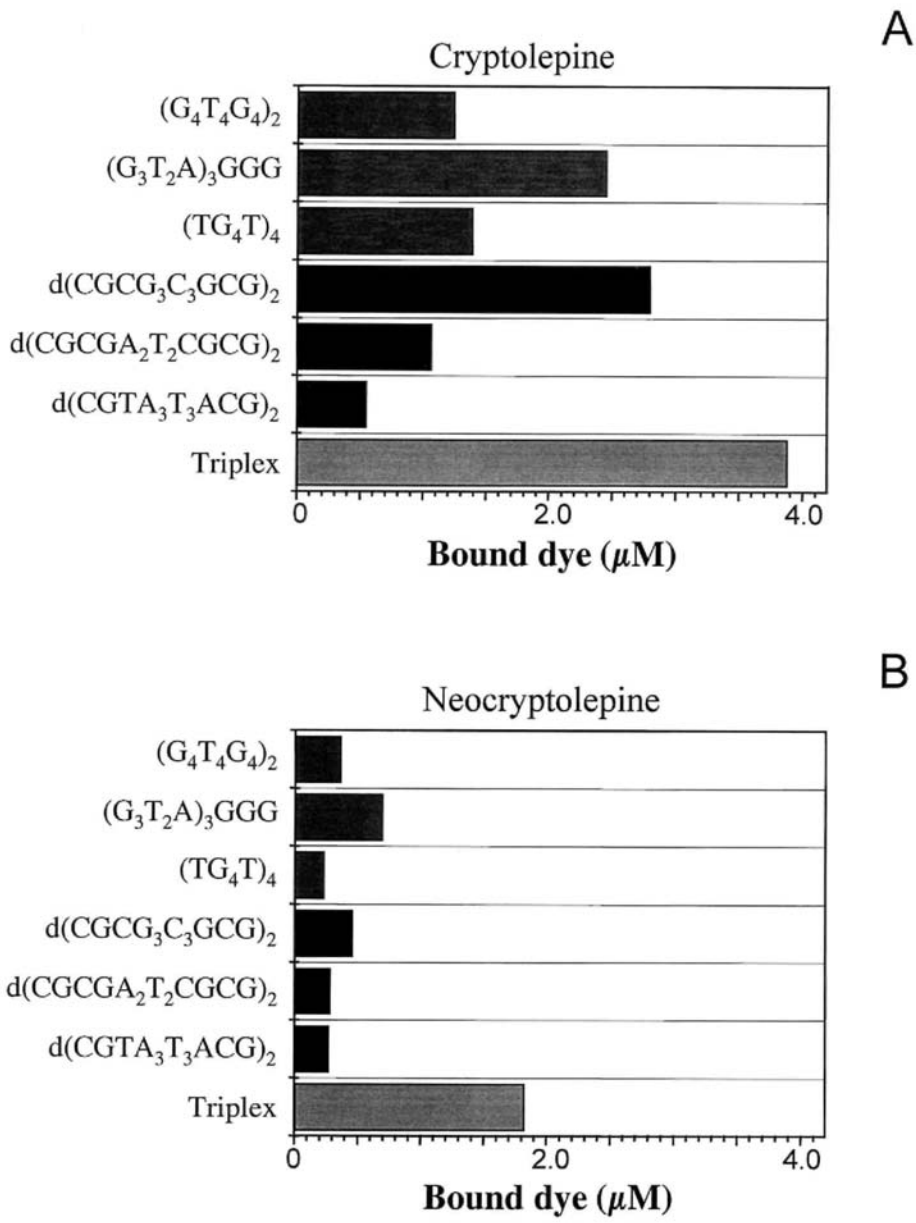


Fig. 4. *Mass spectrometry. Amount of ligand bound (μM) to each monomeric DNA structure determined by ESI-MS. (A) Cryptolepine. (B) Neocryptolepine.*



3.5. Telomerase inhibition

Telomerase inhibition efficiency was measured by a TRAP assay. The non-folded, single-stranded form of the primer is required for optimal telomerase activity and qua-druplex formation has been shown to directly inhibit telomerase elongation in vitro [20]. A growing number of small molecules have been discovered to inhibit telomerase activ ity by inducing and/or stabilizing G-quartet structure [45,46], including l0H-indolo [3,2-b] quinoline derivatives [47]. Therefore, here, we performed a TRAP assay with increasing concentrations (ranging from 1 to 50 μM) of the compound. Cryptolepine inhibits telomerase with an IC_{50} of 9.4 μM (Fig. 8). The presence of an internal control (ITAS) discriminates the inhibition of Taq polymerase during PCR amplification and telomerase elongation. A weak inhibition of the ITAS is detected up to 5 μM before telomerase elongation. It may reflect the preference of cryptolepine for a duplex than a G-quadruplex structure. This IC_{50} (in the 10 μM

range) is relatively modest when compared to the best G4-based telomerase inhibitors described, and reflects a relatively weak interaction with G-quadruplexes. No significant telomerase inhibition is observed with neocryptolepine derivatives (IC_{50} > 40 µM for all the compounds), in agreement with the absence of G-quadruplex stabilization.

***Fig. 5**. Denaturation profiles obtained for the poly(dA)/2poly(dT) triplex in the absence (solid line, triangles) or in the presence of cryptolepine (3 µM, dotted line, circles). Absorbance values were recorded at 260 nm. Experimental conditions: 10 mM sodium cacodylate, 5 mM $MgCl_2$ and 100 mM NaCl (pH 6.5).*

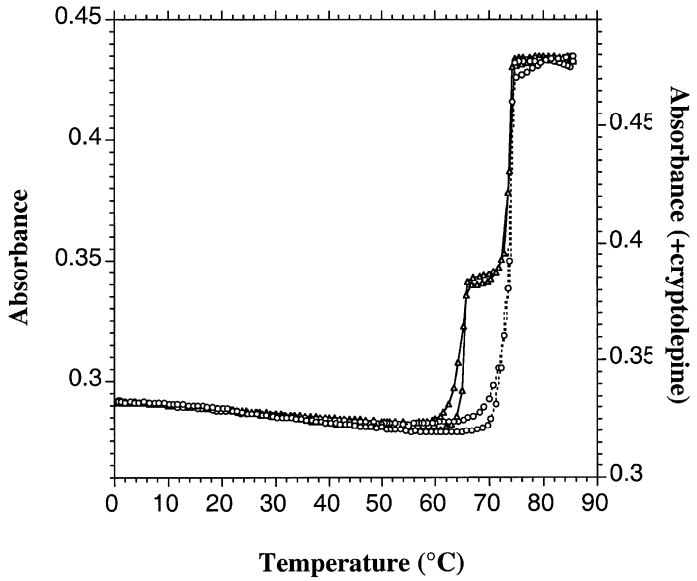


***Fig. 6.** Absorbance differential spectra of cryptolepine in the presence of poly(dT) (triangles), poly(dA)/poly(dT) duplex (dotted line, circles) and poly(dA)/2poly(dT) triplex (squares). Experimental conditions: 10 mM sodium cacodylate, 5 mM $MgCl_2$, 100 mMNaCl (pH 6.2) and 3 µM cryptolepine.*

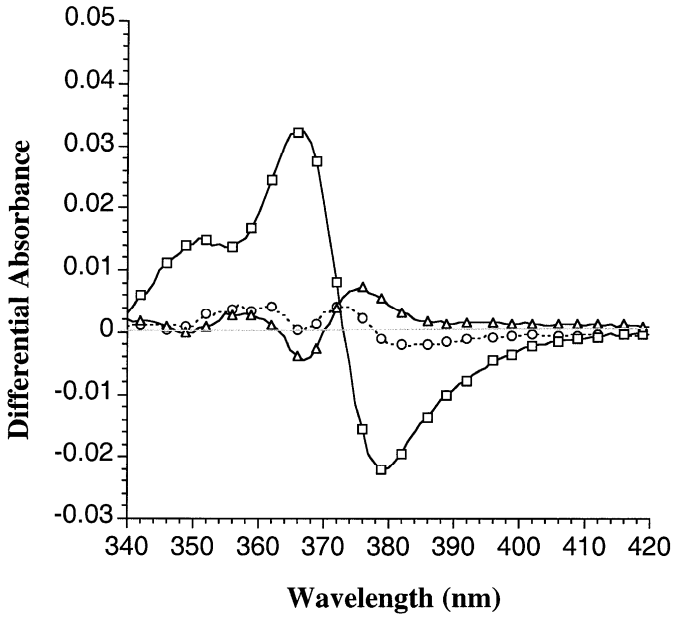


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Fig. 7. *Absorbance titration of cryptolepine by a G4-forming oligonucleotide. (A) Absorbance spectra of 10 µM cryptolepine in a 10 mM sodium cacodylate pH 7.2 buffer with 0.1 M KC1. (B) Quantification at three different wavelengths.*

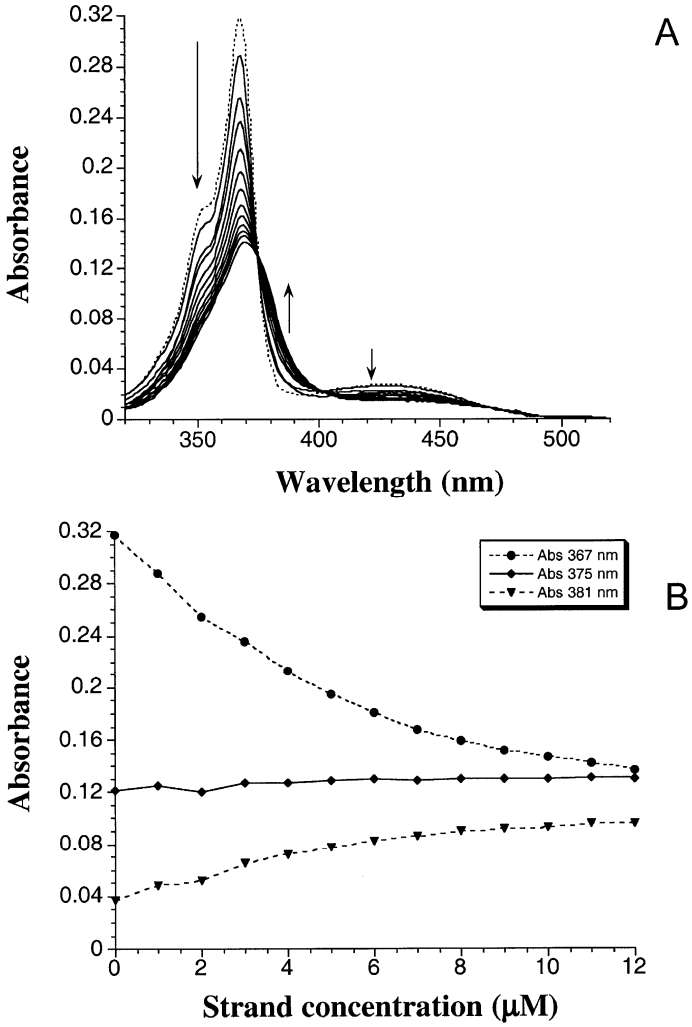
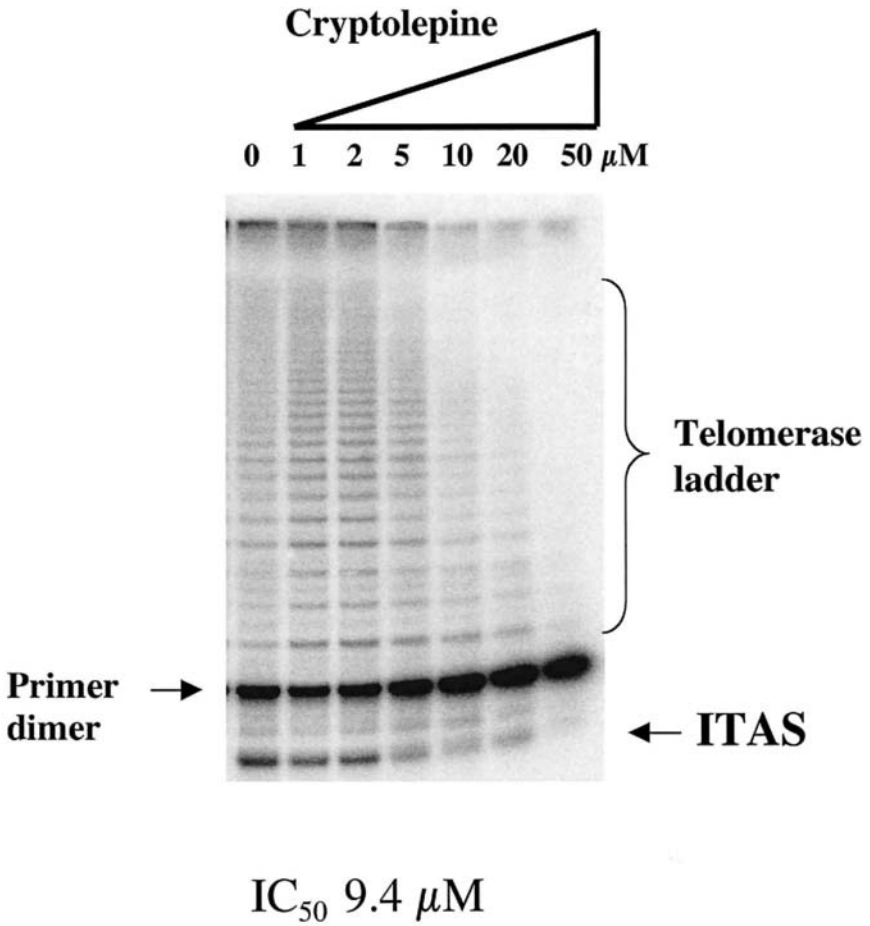


Fig. 8. *Telomerase inhibition by cryptolepine in a TRAP assay. Increasing concentrations of cryptolepine were added in the TRAP mixture in the presence of an internal control (ITAS). TRAP activity was determined with 200 ng of a CHAPS extract of A549 cell line. An IC_{50} of 9.4 µM was determined.*



4. Discussion

In this paper, we have used several methods to study the interaction of the antimalarial drug cryptolepine with unusual DNA structures. This compound binds to G-quadruplexes, as demonstrated by dialysis, mass spectrometry, absorbance titration and FRET studies. However, its binding affinity is very modest. Cryptolepine stabilizes the F21MB quadruplex by 3 °C at 3 µM, whereas the best G4 ligands stabilize by 20 °C or more at 1 µM compound [44]. It is not a potent telomerase inhibitor (IC_{50} = 9.4 µM); the best inhibitors described so far have an IC_{50} of 50 nM or lower [37,44,48-50].

The competition dialysis provided us with data on the affinity of the dye for triplexes, quadruplexes, duplexes and single strands. We confirm the observation that cryptolepine preferentially binds to poly d(GC) rather than to poly d(AT). DNA or RNA single strands are not good substrates for cryptolepine and no fixation on these structures has been observed. Interestingly, oligonucleotides which form an i-motif are cytosine-rich, but are not good substrates for cryptolepine despite its affinity for non-alterning cytosine-rich duplexes. Neocryptolepine exhibited a lower global affinity for DNA than cryptolepine, as shown by equilibrium dialysis and mass spectrometry. This observation may be related to the reduced cytotoxicity of neocryptolepine and its derivatives as compared to cryptolepine [25]. These molecules are developed as selective antimalarial agents and should not exhibit pronounced DNA interactions.

Mass spectrometry may also be used to obtain relative affinities for various structures. To our knowledge, this is the first time the both the techniques (MS and competition dialysis) were compared. These assays gave qualitatively similar results (i.e. preference for triplexes, and global higher binding of cryptolepine as compared

to neocryptolepine). Some differences were nevertheless evidenced. Within the quadru-plex family, equilibrium dialysis suggests roughly equivalent affinities for the antiparallel (22AG) and parallel (24G20) structures, whereas MS results demonstrate a preferential binding to the antiparallel quadruplex ($(GGGTTA)_3GGG$) as compared to the parallel $[TGGGGT]_4$ structure. Further experiments using identical oligomers will be required in order to determine whether these differences are artefactual or caused by different experimental conditions.

Interestingly, cryptolepine derivatives displayed good affinity for triplexes and we carried out further experiments using UV-melting studies and UV-absorbance titration. We show here that cryptolepine is a novel poly(dA)/2poly(dT) triplex ligand leading to a stabilization of 10 °C. Therefore, cryptolepine constitutes a novel triplex stabilizing agent. Several compounds like a tricationic cyanine dye [51] or neomycine [52] have already been described as poly(dA)/2poly(dT) triplex ligands. Interestingly, a structural similarity can be established between cryptolepine and the tetracyclic benzopyridoindole compounds BePI and BgPI which potently stabilize triplex DNA structures [15,53]. The carbazole moiety of cryptolepine, also present in the BePI/BgPI-type compounds as well as in another triplex-stabilizing agent NB506 [29], likely represents a key structural element for triplex binding. This study also adds cryptolepine to the growing list of topoisomerase I or II inhibitors which are also triplex-stabilizers [29,54,55]. The basis of a possible correlation between triplex binding and topoisomerase inhibition is certainly unclear. It might be linked to possible structural similarities between triplex DNA and a specific binding site for cryptolepine within the Topoisomerase-DNA complex.

In conclusion, equilibrium dialysis and mass spectrometry may be used to analyse the structural selectivity of nucleic acid ligands, such as cryptolepine derivatives. Recent reports have shown that some molecules thought to be quadruplex-specific are rather triplex-specific [30]. The cryptolepine derivatives presented here exhibit a significant preference for triplexes over quadruplexes or duplexes.

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