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In vitro anticancer activity and evaluation of DNA duplex binding affinity of phenyl-substituted indoloquinolines

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ABSTRACT

Phenyl-substituted indoloquinolines were studied for their biological activity and their DNA binding affinity. Water-soluble aminoalkyl derivatives were prepared and have shown significant in vitro anticancer activity. Unlike previous reports on the potential role of duplex DNA as target for various indoloquinoline based drugs, duplex UV melting experiments and fluorescence titrations suggest only weak and moderately strong binding of the phenyl-substituted indoloquinolines at 120 mM and 20 mM Na^+ concentrations, respectively. Binding is suggested by ethidium displacement and circular dichroism experiments to be associated with drug intercalation between base pairs.

In recent years, natural and synthetic compounds with the tetracyclic indoloquinoline ring system have attracted growing interest due to their large spectrum of biological activities.[1] The indolo[3,2-*b*]quinoline cryptolepine, a 5N-methylated benzo-δ-carboline, constitutes the major indoloquinoline alkaloid isolated from *Cryptolepis sanguinolenta*. It has been extensively studied for its pharmacological effects revealing, among others, strong antiplasmodial as well as anticancer activities.[2] There is evidence, that the drug interacts with DNA through intercalation at CG-rich sequences and exerts its cytotoxic action through interference with DNA replication and transcription and the stabilization of topoisomerase II–DNA covalent complexes.[3] In fact, an X-ray structure of a cryptolepine–DNA complex demonstrated intercalation into DNA at nonalternating CC sites.[4] Recent studies on the biological effects and DNA binding properties of other natural and synthetic indoloquinolines including different indolo[3,2-*c*]quinoline derivatives[5] have confirmed their remarkable biological potency with promising antiproliferative activities towards various cancer cells in some cases.[5–8] Experimental evidence has suggested cellular DNA to be a primary target for many of these drugs, associated with their intercalation into the DNA double helix followed by the inhibition of DNA synthesis.[1]

We have recently synthesized a novel indolo[3,2-*b*]quinoline with an unfused carboxy-functionalized phenyl ring at C11 and covalently attached it to the 5′- or 3′-end of an amino-modified single-stranded oligonucleotide. Binding studies employing these oligonucleotide–indoloquinoline conjugates have shown, that the attached ligand has only a moderate impact on the stability of double-stranded DNA but is able to effectively promote triplex formation, presumably via its intercalation at the triplex–duplex junction.[9–11] Prompted by their close structural similarity with other antiproliferative indoloquinoline drugs like cryptolepine, we decided to evaluate the as yet unknown cytotoxic activity for these ligands in their free form. Also, in light of the popular belief that genomic DNA constitutes a primary target for a majority of indoloquinoline drugs, their specific DNA duplex binding was examined in more detail for the first time. As a prerequisite for an evaluation of DNA binding–activity relationships, we have synthesized an aminoalkyl derivative of the benzo-annulated δ-carboline for increased water solubility.

Starting with 4,9-dimethyl or 4,9-dimethoxy-substituted 11-(4-methyloxycarbonylphenyl)-10*H*-indolo[3,2-*b*]quinoline **1a** and **1b**,[12] the methyl esters were initially hydrolyzed under alkaline conditions. The resulting carboxylic acids **2a** and **2b** were dissolved in dry DMF and activated by addition of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (DCI) and *N*-hydroxysuccinimide (NHS). The purified NHS esters **3a** and **3b** were subsequently coupled with 2-dimethylaminoethylamine in DMF at room temperature to give the amidation products **4a** and **4b** in good yields (Scheme 1).[13,14]

The indoloquinolines **4** were evaluated for antiproliferative activity together with the poorly water soluble parent compounds **1** in 6 human cancer cell lines; two bladder (5637, RT-4), two lung

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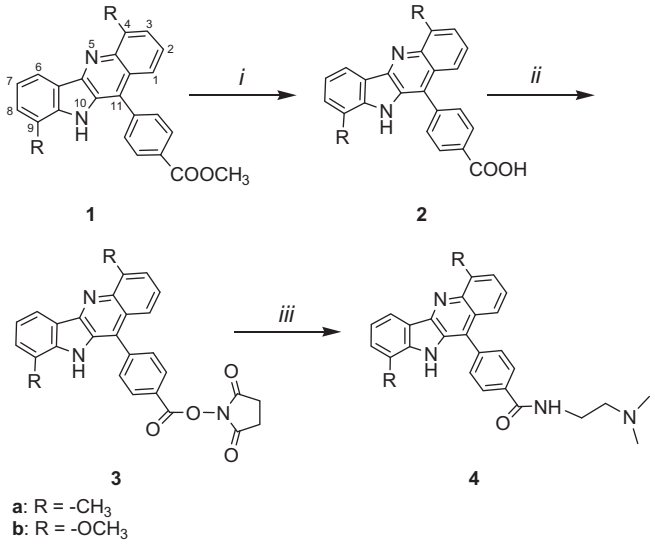

Scheme 1. Synthesis of dimethylaminoethylamine-modified indoloquinolines **4a** and **4b**. Reagents and conditions: (*i*) KOH, MeOH; (*ii*) NHS, DCI, DMF, rt; (*iii*) 2-dimethylaminoethylamine, DMF, rt, 2 h.

(A-427 and LCLC-103H), one pancreatic (DAN-G) and one breast (MCF-7) cancer cell type. Cell growth inhibition was determined with a well established microtiter assay based on staining cells with crystal violet as described in detail elsewhere.[15] Cells were treated for 96 h with five concentrations at two-fold dilutions in DMSO. IC_{50} values are the concentrations of test substance that inhibit cell growth by 50% compared to untreated controls. Both *N,N*-dimethylamino-modified compounds **4a** and **4b** showed significant antiproliferative potency in all tested cell lines with IC_{50} values ranging from 1.7 to 4.2 μM (Table 1). There was no noticeable change of the in vitro cytotoxicity when replacing the methyl by methoxy substituents. In contrast, the methyl ester derivatives **1a** and **1b** exhibit IC_{50} values >10 μM for all cell lines, attributable to their low effective concentration as a result of poor solubilities in the cell culture medium.

UV thermal melting experiments were used for a first assessment of the DNA binding affinity of the compounds. Any change in the melting temperature ΔT_m of the duplex upon ligand addition is a measure of its relative duplex versus single strand stabilization and consequently of its specific duplex binding. Three dodecamer duplexes were used as DNA targets in the binding studies of the indoloquinoline derivatives (Fig. 1). All duplexes were chosen not to be self-complementary to avoid any ambiguities in case of potential hairpin formation under appropriate conditions. Also, to allow for a sequence discrimination of the ligands, duplexes **DG** and **DA** comprise a central all-CG and all-AT base pair tract, respectively. In contrast, **DM** features a randomly mixed sequence along the entire duplex. Note, that each of the possible 16 base pair steps is incorporated in at least one of the duplexes.

Table 1
IC_{50} values (μM) of **1a**, **1b**, **4a** and **4b** for selected cancer cell lines after 96 h treatment[a]

	5637	RT-4	A-427	LCLC-103H	DAN-G	MCF-7
1a	>20	>20	>20	>20	>20	13.8 ± 9.0
1b	11.0 ± 4.6	>20	16.6 ± 12.3	>20	>20	>20
4a	3.2 ± 1.2	4.2 ± 1.1	3.2 ± 1.8	3.7 ± 0.2	2.6 ± 0.1	3.0 ± 0.4
4b	2.1 ± 0.4	3.6 ± 0.4	1.7 ± 0.5	3.2 ± 0.8	3.6 ± 0.2	2.9 ± 0.3

[a] Average values with standard deviations from 4 to 5 independent measurements.

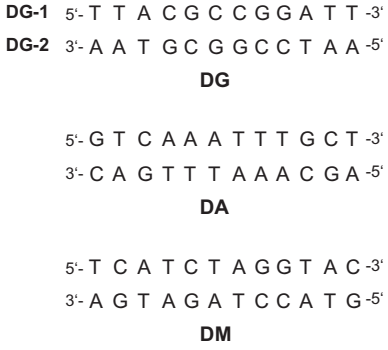

Figure 1. Duplexes **DG**, **DA** and **DM**.

Melting temperatures are summarized in Table 2 for the three duplexes without and with the indoloquinolines **4a** and **4b**, added in a 5:1 molar excess over the duplex. Apparently, in a buffer containing 100 mM NaCl hardly any changes in duplex melting are detected. These data suggest either no ligand binding under these conditions or binding of similar strength to both double-stranded and single-stranded DNA.

The two indoloquinoline ligands carry positive charges due to amino side chain protonation at neutral pH and hence a significant electrostatic contribution is expected if there are any binding processes. The effect of salt on the DNA–ligand interaction was studied in melting experiments using a 20 mM cacodylate buffer without any additional NaCl. Whereas the denaturation temperature of all duplexes in the absence of ligand decreases by more than 10 °C as expected by the lower salt concentration, the duplex binding affinity increases as indicated by the noticeable ligand-mediated increase in melting temperature ΔT_m under these conditions (Table 2). The associated more negative free energy of complex formation may be largely attributed to a favorable polyelectrolyte contribution associated with the release of counterions from the DNA duplex when interacting with the charged ligand. Compound **4b** with its two methoxy substituents tends to be slightly more stabilizing under low-salt conditions when compared to **4a** with two methyl groups. On the other hand, the ligand-induced increase in T_m exhibits no pronounced sequence dependence and differences in ΔT_m for the three duplexes can be attributed to experimental uncertainties and to differences in their absolute melting temperatures where binding is monitored.

Given the closely similar binding behavior of **4a** and **4b** towards the various duplexes, indoloquinoline derivative **4b** and the high-melting duplex target **DG** with its central CG tract, known to be a preferred target of the related cryptolepine, were employed for more detailed binding studies. The indoloquinolines are

Table 2
UV melting temperatures T_m (°C) of duplexes with and without ligand[a]

	DG		DA		DM	
	T_m	ΔT_m	T_m	ΔT_m	T_m	ΔT_m
In 0.02 M cacodylate, 0.1 M NaCl, pH 7						
[b]	52.5 ± 0.9	—	44.8 ± 0.2	—	40.1 ± 0.2	—
4a	51.6 ± 0.4	−0.9	45.1 ± 0.5	+0.3	40.3 ± 0.5	+0.2
4b	52.0 ± 0.3	−0.5	45.3 ± 0.4	+0.5	40.6 ± 0.2	+0.5
In 0.02 M cacodylate, pH 7						
[b]	40.7 ± 0.9	—	32.8 ± 0.2	—	29.7 ± 1.0	—
4a	41.5 ± 0.5	+0.8	34.3 ± 0.1	+1.5	30.7 ± 0.1	+1.0
4b	42.4 ± 0.9	+1.7	35.3 ± 0.3	+2.5	32.8 ± 0.2	+3.1

[a] Average T_m values with standard deviations from 3 independent measurements on a mixture of duplex (2 μM) and ligand (10 μM).
[b] Duplex without ligand.

fluorescent compounds and **4b** shows fluorescence emission maxima at about 500 and 480 nm when excited at 350 nm in aqueous buffer. As shown in Figure 2, titrating the duplex to a low-salt buffer solution of **4b** results in a moderate quenching of fluorescence. This points to an intercalative mode of binding based on the previously observed fluorescence quenching of cryptolepine when titrated with DNA[3] and of a corresponding TFO conjugated **4b** derivative, thought to act as intercalator upon triplex formation with a target duplex.[11] It should be mentioned, that the ligand was titrated in the presence of an excess single-stranded oligonucleotide. Drug fluorescence was shown in separate experiments to be significantly influenced in the presence of any arbitrary oligonucleotide even when single-stranded, indicating largely nonspecific outer binding through charge interactions with the polyanionic DNA backbone (not shown). With saturating amounts of unstructured single-stranded DNA, the impact of these nonspecific electrostatic interactions on the drug fluorescence is mostly eliminated and specific duplex binding determines changes in the fluorescence emission. Fitting of the titration data with the total concentration of duplex DNA expressed in terms of base pairs (bp) and employing a model with equal and independent binding sites yields a K_a^{app} of 3.8×10^4 M (bp)$^{-1}$ (see inset Fig. 2). Note that this binding constant, related to a base pair, serves as a good measure of the duplex binding affinity given the difficulty of extracting additional binding stoichiometries or binding site sizes for weak or only moderately strong binding phenomena.

Competitive ethidium displacement studies give additional information on the duplex association of the phenyl–indoloquinolines.[16] Figure 3 shows the fluorescence intensity of ethidium bromide (EB) in a solution with duplex as a function of added **4b**. Generally, displacement of the strongly fluorescent EB intercalator from the duplex is accompanied by a loss of fluorescence. This fluorescence quenching can be used to assess the relative binding in terms of a C_{50} value, that is, the ligand concentration that causes a 50% reduction in the fluorescence of the ethidium–DNA complex. However, addition of **4b** results in an initial EB fluorescence enhancement before a decrease in fluorescence due to ethidium displacement from the DNA intercalation sites is observed. This fully reproducible behavior must be attributed to an initial noncompetitive binding process, followed by EB displacement at higher drug concentrations. The observed C_{50} values of 93 μM and 118 μM based on the maximum and initial EB fluorescence, respectively, indicate a poor EB displacement efficiency due to rather weak intercalative DNA binding of the indoloquinoline in line with the UV and fluorescence titration data.

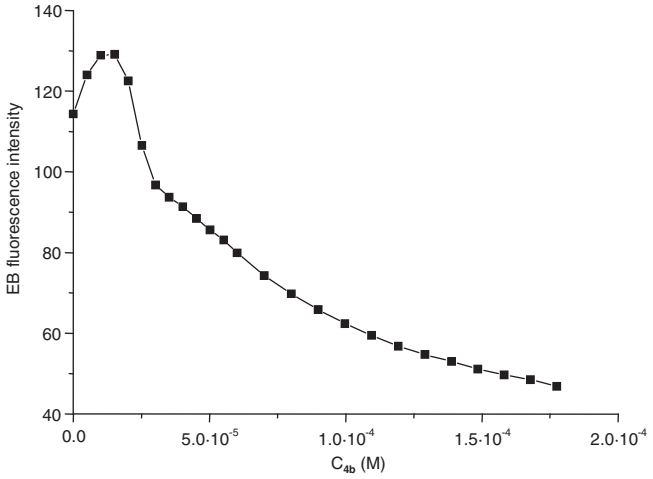

Figure 3. Competitive displacement experiment in 0.02 M sodium cacodylate, pH 7. The fluorescence intensity decrease of ethidium bromide (1.26 μM) in a mixture with duplex **DG** (0.75 μM in base pairs) is plotted against the concentration of added **4b** (0–187 μM).

A weak intercalative binding mode is further corroborated by the circular dichroism spectra of duplex in the absence and presence of **4b** as shown in Figure 4. In the absence of the ligand, the CD spectrum of **DG** exhibits the CD signature of a typical B-type duplex with a positive band near 275 nm and a negative band near 250 nm.[17] Adding **4b** results in only minor changes with a small increase of both positive and negative CD amplitudes. In addition, an induced negative CD effect (ICD) of very low intensity and completely absent in the corresponding CD spectrum of single-stranded oligonucleotide in the presence of **4b** (not shown) can be detected in the wavelength range corresponding to the indoloquinoline absorption above 300 nm (see inset Fig. 4). Such an ICD suggests a weak intercalative mode of binding within the asymmetric DNA environment.[18]

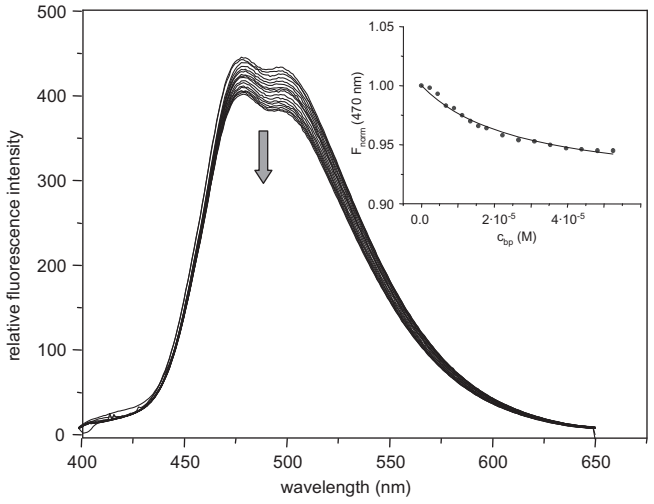

Figure 2. Fluorescence emission spectra during titration of duplex **DG** (0–4.5 μM) at 25 °C to indoloquinoline derivative **4b** (0.75 μM) in the presence of excess single-stranded **DG-2** (21 μM) in 0.02 M cacodylate buffer, pH 7; the inset shows a least-squares fit of the **4b** fluorescence intensity at 470 nm. The arrow indicates the change of **4b** fluorescence during DNA addition.

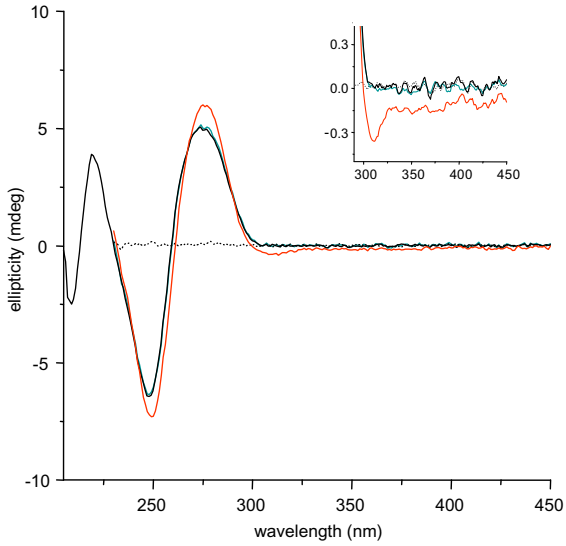

Figure 4. CD spectra of free **4b** (10 μM, black dotted line), **DG** (2 μM, solid black line), their summed spectra (blue line) and a mixture of **DG** and **4b** (1:5 molar ratio, red line) at 25 °C in 0.02 M sodium cacodylate, pH 7.0. The inset shows a vertical expansion of the drug long-wavelength absorption region.

In summary, the methyl and methoxy-substituted phenyl–indoloquinolines exhibit significant cytotoxic activities when their solubility in buffer solution is sufficiently increased through the introduction of a cationic aminoalkyl chain. However, in contrast to previous reports, suggesting that genomic DNA plays an important role in the biological activity of many indoloquinoline drugs, our binding studies only suggest weak to medium intercalative interactions of the phenyl–indoloquinolines, questioning a direct participation of double-helical DNA in their considerable biological activity. This may simply be a consequence of their unique structural and physical properties, in particular imparted by the unfused phenyl substituent at C11, favoring more extended intercalation sites as, for example, provided by the base triads in DNA triplexes. Nevertheless, the notion of duplex DNA as being a major target may also be revisited for some of the known cytotoxic indoloquinoline drugs in light of the evidence presented here.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.02.088.

References and notes

1. Lavrado, J.; Moreira, R.; Paulo, A. *Curr. Med. Chem.* **2010**, *17*, 2348.
2. Cimanga, K.; De Bruyne, T.; Pieters, L.; Vlietinck, A. J.; Turger, C. A. *J. Nat. Prod.* **1997**, *60*, 688.
3. Bonjean, K.; De Pauw-Gillet, M. C.; Defresne, M. P.; Colson, P.; Houssier, C.; Dassonneville, L.; Bailly, C.; Greimers, R.; Wright, C.; Quetin-Leclercq, J.; Tits, M.; Angenot, L. *Biochemistry* **1998**, *37*, 5136.
4. Lisgarten, J. N.; Coll, M.; Portugal, J.; Wright, C. W.; Aymami, J. *Nat. Struct. Biol.* **2002**, *9*, 57.
5. Lu, C.-M.; Chen, Y.-L.; Chen, H.-L.; Chen, C.-A.; Lu, P.-J.; Yang, C.-N.; Tzeng, C.-C. *Bioorg. Med. Chem.* **2010**, *18*, 1948.
6. Arzel, E.; Rocca, P.; Grellier, P.; Labaeïd, M.; Frappier, F.; Guéritte, F.; Gaspard, C.; Marsais, F.; Godard, A.; Quéguiner, G. *J. Med. Chem.* **2001**, *44*, 949.
7. Hu, X.-W.; Chien, C.-M.; Yang, S.-H.; Lin, Y.-H.; Lu, C.-M.; Chen, Y.-L.; Lin, S.-R. *Cell Biol. Toxicol.* **2006**, *22*, 417.
8. Van Miert, S.; Hostyn, S.; Maes, B. U. W.; Cimanga, K.; Brun, R.; Kaiser, M.; Mátyus, P.; Dommisse, R.; Lemière, G.; Vlietinck, A.; Pieters, L. *J. Nat. Prod.* **2005**, *68*, 674.
9. Todorović, N.; Thi Bich Phuong, N.; Langer, P.; Weisz, K. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 1647.
10. Eick, A.; Xiao, Z.; Langer, P.; Weisz, K. *Bioorg. Med. Chem.* **2008**, *16*, 9106.
11. Eick, A.; Riechert-Krause, F.; Weisz, K. *Bioconjug. Chem.* **2010**, *21*, 1105.
12. Langer, P.; Anders, J. T.; Weisz, K.; Jähnchen, J. *Chem. Eur. J.* **2003**, *9*, 3951.
13. *Spectroscopic data of* ***4a***: ^{1}H NMR (600 MHz, CD_3OD): δ (ppm) = 8.27 (d, J = 7.7 Hz, 1H, ArH), 8.05 (d, J = 8.7 Hz, 2H, ArH), 7.62 (d, J = 8.7 Hz, 2H, ArH), 7.48 (d, J = 8.7 Hz, 1H, ArH), 7.44 (d, J = 7.7 Hz, 1H, ArH), 7.30 (d, J = 7.2 Hz, 1H, ArH), 7.25 (t, J = 7.5 Hz, 1H, ArH), 7.14 (t, J = 7.2 Hz, 1H, ArH), 3.55 (t, J = 7.2 Hz, 2H, $-CH_2-$), 2.91 (s, 3H, CH_3-), 2.60 (t, J = 6.8 Hz, 2H, $-CH_2-$), 2.45 (s, 3H, CH_3-), 2.30 (s, 6H, 2 CH_3-); UV–vis: ε_{260} = 24,247 ± 793 L mol^{-1} cm^{-1}; ε_{348} = 9893 ± 515 L mol^{-1} cm^{-1}; ε_{408} = 4329 ± 252 L mol^{-1} cm^{-1}; HRMS (ESI-TOF): m/z for $C_{28}H_{29}N_4O$ $[M+H]^+$ calcd 437.2336, found 437.2331.
14. *Spectroscopic data of* ***4b***: ^{1}H NMR (600 MHz, CD_3OD): δ (ppm) = 8.19 (d, J = 8.4 Hz, 1H, ArH), 8.03 (d, J = 8.4 Hz, 2H, ArH), 7.62 (d, J = 7.9 Hz, 2H, ArH), 7.31 (t, J = 8.4 Hz, 1H, ArH), 7.22 (d, J = 8.4 Hz, 1H, ArH), 7.17 (t, J = 8.4 Hz, 1H, ArH), 7.08 (d, J = 7.3 Hz, 1H, ArH), 7.04 (d, J = 7.9 Hz, 1H, ArH), 4.08 (s, 3H, CH_3-), 3.91 (s, 3H, CH_3-), 3.57 (t, J = 6.7 Hz, 2H, $-CH_2-$), 2.66 (t, J = 6.2 Hz, 2H, $-CH_2-$), 2.35 (s, 6H, 2 CH_3-); UV–vis: ε_{260} = 21,596 ± 517 L mol^{-1} cm^{-1}; ε_{348} = 5315 ± 114 L mol^{-1} cm^{-1}; ε_{408} = 3467 ± 97 L mol^{-1} cm^{-1}; HRMS (ESI-TOF): m/z for $C_{28}H_{29}N_4O_3$ $[M+H]^+$ calcd 469.2234, found 469.2246.
15. Bracht, K.; Boubakari; Grünert, R.; Bednarski, P. J. *Anticancer Drugs* **2006**, *17*, 41.
16. McConnaughie, A. W.; Jenkins, T. C. *J. Med. Chem.* **1995**, *38*, 3488.
17. Kypr, J.; Kejnovská, I.; Renčiuk, D.; Vorlíčková, M. *Nucleic Acids Res.* **2009**, *37*, 1713.
18. Tuite, E.; Nordén, B. *J. Am. Chem. Soc.* **1994**, *116*, 7548.