**Abstract:** Indoloquinoline alkaloids isolated from natural sources are known for their broad spectrum of biological activities. The cytotoxic action of some derivatives is thought to arise from their interaction with genomic DNA driven by favorable stacking interactions of the intercalated indoloquinoline skeleton with the DNA base pairs. In addition to double-helical DNA, indoloquinoline derivatives have also been found to bind DNA triplexes as well as G-quadruplexes with high affinity, opening interesting perspectives towards the development of indoloquinoline-based DNA binding ligands with numerous potential applications in therapeutics, diagnostics and microbiology. The present paper presents an overview of studies dealing primarily with the binding of indoloquinoline analogs to double-helical, triple-helical or quadruplex targets. Available thermodynamic and structural data that also include few experimentally determined three-dimensional structures of DNA-drug complexes highlight key elements for an improved binding affinity but also important structural aspects for the design of structure-selective analogs, prerequisite for many potential applications.

**Keywords:** DNA duplex; DNA triplex; G-quadruplex; indoloquinoline.

**Introduction**

Tetracyclic indoloquinolines have attracted considerable interest in the recent past due to their broad spectrum of biological activities and their binding to various types of nucleic acids. The four parent isomers indolo[2,3-b]quinoline, indolo[2,3-c]quinoline, indolo[3,2-c]quinoline and indolo[3,2-b]quinoline may also be regarded as benzoannelated α-, β-, γ- and δ-carbolines with a tricyclic pyridoindole core structure (Figure 1A). Natural indoloquinoline alkaloids are almost exclusively isolated from the West African shrub *Cryptolepis sanguinolenta* and include the unsubstituted indolo[3,2-b]quinoline also named quindoline and its N5-methyl-substituted derivative cryptolepine [1–4] (Figure 1B). Extracts of the plant have been used for a long time as a traditional medicine in Central and West Africa against a variety of disorders including malaria. In addition to its antimalarial activity, cryptolepine itself has been shown to also exert antibacterial, antifungal and antitumor activities [5–12]. Two other N5-methylated isomers, that is, the linear 5-methyl-indolo[2,3-b]quinoline or neocryptolepine and the angular 5-methyl-indolo[3,2-c]quinoline also termed isocryptolepine were later isolated as minor alkaloids from the same plant [13–15]. Isoneocryptolepine or N5-methyl-indolo[2,3-c]quinoline, the remaining isomer of the N5-methylated indoloquinoline series was only synthesized recently [16]. Curiously, derivatives of the latter isomer have not been found in nature, although its β-carboline substructure directly derives from tryptophan and β-carboline alkaloids are the most prevalent tricyclic carbolines extracted from natural sources.

Studies on the biological activity of various indoloquinoline derivatives revealed their potency to serve as lead compounds in the fight against various infectious diseases as well as against cancer. Although our knowledge about specific cellular targets for their often multiple mechanisms of action within a cell is still limited, their DNA binding affinity observed *in vitro* indicates DNA to be a major potential target for many indoloquinolines. Similar to other drugs possessing a polycyclic ring system such as 9-aminoacridine or the antineoplastic agent ellipticine [17], cytotoxic effects of cryptolepine were reported to be a result of drug intercalation between DNA base pairs followed by inhibition of topoisomerase II [18]. Note that the pyrido[4,3-b]carbazole ring structure of ellipticine is closely related to the indolo[3,2-b]quinoline skeleton and can be viewed as its 3-aza-5-deaza analog with interchanged C and N atoms in positions 3 and 5 (Figure 1C).

Although cytotoxicities and the affinity for nucleic acids are often strongly correlated for DNA binding ligands, it should be borne in mind that drugs must
overcome various barriers in addition to their DNA binding to be biologically active. Important aspects include their cytoplasmic and nuclear membrane permeability, their metabolic stability and their capability after DNA binding to inhibit enzymes important for transcription and replication such as topoisomerases I and II. Consequently, cytotoxic effects may sometimes show only poor correlations to DNA binding parameters even in the case of drugs acting through complex formation with DNA. However, their DNA binding ability remains a prerequisite for their physiological activity and of their potential use as, for example, anticancer agents. By contrast, the antimalarial potency of cryptolepine seems to at least partially derive from inhibition of hemozoin formation in malaria parasites without major contributions from DNA binding processes. Unfortunately, the toxicity of cryptolepine originating from its ability to intercalate into DNA and to inhibit topoisomerase II restricts its use as an antimalarial drug. Here, derivatives with reduced DNA binding affinity associated with less cytotoxic side effects are needed for the development of more selective antimalarial drugs.

Recently, biophysical studies on indoloquinolines have shown their high-affinity binding not only to double-stranded genomic DNA but also to triple-stranded and quadruplex structures [19]. This recognition of other DNA secondary structures extends potential targets for the indoloquinolines and offers possibilities for more selective interactions with the DNA and a more effective combat of diseases. It also expands perspectives beyond their use as lead structures for the development of more potent antimicrobial or anticancer drugs to promising candidates for the development of specific DNA binding ligands in general. Such ligands are urgently needed for numerous applications in biotechnology, microbiology or diagnostics. Given the fundamental importance of DNA binding affinity and selectivity in current drug development but also for non-medicinal applications, the present review will mostly focus on the DNA recognition of the indoloquinolines without dealing in more detail with specific biological effects or synthetic methodologies. For a comprehensive account of in vitro and in vivo biological activities as well as of the synthesis of indoloquinolines, please see [20] and [21].

**General considerations**

Indoloquinolines comprise a bicyclic substructure with a \( \pi \)-electron rich pyrrole ring fused to a \( \pi \)-electron deficient pyridine heterocycle. Owing to their two-ring nitrogens, indoloquinolines may occur in two different tautomeric
forms as illustrated in Figure 2A for the indolo[3,2-b]quinoline. Whereas simple pyrrolopyridines have been shown to undergo excited state tautomerization [22], 5H-pyrido tautomers of indoloquinolines are expected to be energetically disfavored in the ground state and H-pyrrolo tautomers should be considered the active species [23]. Acid base properties of indoloquinolines are also important determinants when evaluating their molecular interactions. Clearly, annelation of a pyrrole and pyridine ring is expected to affect protonation equilibria when compared with simple heterocycles. Experimental pK_a values for protonation at the two-ring nitrogen atoms as shown in Figure 2B are summarized in Table 1. They were either obtained spectrophotometrically [23–26] or by 1H NMR spectroscopy [27–29]. For comparison but also due to incomplete experimental data for all parent isomers in aqueous solution, pK_a values of the indoloquinolines were complemented with data on the tricyclic carboline substructures. For the latter, pK_a values cluster in a relatively narrow range between 14.0 and 15.1. By contrast, N-methylation at the pyridine nitrogen considerably increases acidity for the cryptolepines. With a pK_a of 7.1, neocryptolepine is already significantly deprotonated under neutral solution conditions. However, the other three N-methylated isomers are mostly protonated and thus carry a net positive charge if not dissolved in a strongly alkaline solution.

Interestingly, pK_a values for the pyridine nitrogen protonation in the carboline series exhibit large variations depending on the relative position of the two endocyclic nitrogens. Whereas the α-carboline isomer with its two neighboring N atoms is mainly present in the form of its free base, the γ-carboline is significantly protonated under physiological conditions. The higher pK_a of the latter may be explained by an efficient push-pull interaction between the pyrrole and the protonated γ-positioned pyridine nitrogen. Benzo-annelation of the carbolines may noticeably affect acid base properties in the resulting indoloquinolines as shown by an increase of 1.4 in the pK_α for benzo-α-carboline or by a decrease of 1.1 in the pK_α for benzo-δ-carboline (Table 1). However, pK_α values for parent indoloquinolines can be expected in the range 4–8 and will thus be prone to N5-protonation/deprotonation under physiological conditions. In the following, N5-alkylated indoloquinoline derivatives are represented.
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in their protonated form with a quaternized and positively charged pyrido nitrogen, whereas analogs without N5-alkylation are depicted as neutral species. It should be noted, however, that the protonation state of the predominant species may differ depending on the specific pKₐ value and on the buffer conditions employed.

Based on their planarity and molecular dimensions, tetracyclic indoloquinolines may be considered typical DNA intercalating agents. They clearly lack the crescent-shaped geometry and inherent flexibility to allow for an isohelical conformation, characteristic of many minor groove binding ligands. By contrast, stacking interactions upon intercalation depend on the overlap of the ligand π-electron system with the purine and pyrimidine bases of adjacent base pairs. As shown in Figure 3, a nearly maximum overlap is expected for the indolo[3,2-b]quinoline scaffold when strictly oriented parallel to the long axis of Watson-Crick GC or AT base pairs.

Although important, the overall geometry of the ligand only partially contributes to its intercalating ability. The enthalpy of intercalation involves permanent dipole-dipole interactions as well as dispersion forces and thus also depends on the magnitude and orientation of dipole moments and on the polarizability of the intercalator. Even lacking substituents, the dipole moments are different for the four indoloquinoline positional isomers and are expected to result in different potential stacking interactions. Substituents on the tetracyclic ring system may affect intercalation in various ways. Depending on their position and on their electron-releasing or electron-withdrawing effects they will invariably change the electronic distribution and thus dipole moments within the ring system. Also, the substituent itself may directly interact through ionic, dipolar or van der Waals interactions when positioned in one of the nucleic acid grooves confined by the polyanionic sugar-phosphate backbone. Additionally, substituents may significantly change pKₐ values. If the ligand is subject to protonation equilibria within the pH range examined as expected for the indoloquinolines (vide supra), this can result in a considerably altered degree of protonation for the intercalating heterocyclic compound. It should also be noted that the extent of protonation for a DNA-bound ligand L may significantly deviate from its protonation state when free in solution. Binding and protonation processes are connected according to sequential equilibria.

\[
\begin{align*}
\text{L} + \text{DNA} + \text{H}^+ & \rightleftharpoons \text{L} + \text{DNA} + \text{H}^+ \quad K_{\text{ass}(L)} \\
\text{LH}^+ + \text{DNA} & \rightleftharpoons \text{LH}^+ + \text{DNA} \quad K_{\text{ass}(\text{LH}^+)}
\end{align*}
\]

This yields an apparent \( K_{\text{app}} \) for the bound ligand:

\[
K_{\text{app}} = \frac{c(\text{L} \times \text{DNA})c(\text{H}^+)}{c(\text{LH}^+ \times \text{DNA})} = K_{\text{ass}(\text{L})}/\frac{K_{\text{ass}(\text{LH}^+)}}{K_{\text{ass}(\text{L})}}.
\]

Clearly, \( K_{\text{app}} \) will deviate from the true \( K_{\text{ass}} \) of the free ligand in solution if the free energy of binding differs for protonated and unprotonated species.

Finally, the rearrangement and release of water molecules from solvation/desolvation effects is strongly influenced by changes in the exposed hydrophobic surface area upon complex formation and may lead to significant entropic contributions to the free energy of ligand binding. Taken together, identifying the potential interactions that contribute to a particular association event will in many cases enable the rationalization of relative binding affinities and thus of a rational development of more effective ligands. However, a quantitative description or reliable prediction of DNA interactions for a specific ligand still suffers from the rather complex interplay between the various effects operative upon binding.

**Binding of indoloquinolines to duplex DNA**

**Indolo[3,2-b]quinolines**

Activity measurements for benzo-δ-carbolines with alkyl, phenyl and chloro substituents at C11 demonstrated the importance of N5-methylation for their cytotoxicity [30]. Thus, cryptolepine was shown to be 30 times more cytotoxic on cancer KB cells than quindoline. In comparison, cytotoxicity was only slightly influenced by 11-alkyl substituents. Although no DNA binding data were reported for these studies, cytotoxic activity against mammalian cells is

![Figure 3](image-url) Indolo[3,2-b]quinoline (yellow) superimposed on a GC Watson-Crick base pair (gray).
suggested to depend on drug-DNA interactions and point to a stronger DNA binding for the cryptolepine series.

Owing to its good availability and its use as a lead compound for synthetic antiplasmodial agents, cryptolepine being the major indoloquinoline alkaloid extracted from Cryptolepis plants has probably been the most widely studied indoloquinoline derivative with respect to its DNA binding ability. Although the molecular basis for each of its various biological activities is far from completely understood, binding studies in the past years have provided strong evidence that genomic DNA constitutes its major target and DNA binding may be largely responsible for the cytotoxic effects exerted by the drug. Using a combination of spectroscopic techniques such as absorption and fluorescence spectroscopy as well as circular dichroism (CD) and electric linear dichroism, strong intercalative binding of the alkaloid to DNA was demonstrated [18, 31]. Absorption spectra of the drug during the course of titration with calf thymus DNA resulted in typical bathochromic and hypochromic changes of the absorption bands and the presence of isosbestic points. Although the latter strongly suggest a two-state equilibrium with only one defined binding site for the indoloquinoline, both absorption and fluorescence titration data could only be satisfactorily fitted employing a two-site model with two independent binding sites. With an apparent association constant of $K_a = 3 \times 10^6$ M$^{-1}$ determined at pH 6.5 under conditions of low ionic strength, the high-affinity binding constant was found to be approximately two orders of magnitude higher compared with the additional low-affinity binding. Also, DNAase I footprinting experiments revealed a preferential binding of cryptolepine to GC-rich sequences and such sequence selectivity was later corroborated by competition dialysis experiments, pointing to non-alternating CC$\times$GG rather than CG$\times$CG or GC$\times$GC steps as favored intercalation sites [32].

A crystal structure of the d(CCTAGG)$_2$ B-type hexamer duplex complexed with cryptolepine confirmed drug intercalation at the two CC$\times$GG non-symmetric base pair steps [32, 33]. As shown in Figure 4, the drug is aligned with its major axis parallel to the base pair long axis and penetrates deeply into the helical stack. The almost perfect fit of the drug sandwiched between the neighboring CG base pairs maximizes stacking interactions. With the fused benzene ring of the indole moiety stacked between the two cytosines and the quinoline portion of the molecule stacked between the two guanines, the positively charged cryptolepine N5 nitrogen is placed between the 6-carbonyl oxygens of the two adjacent guanines in the major groove. Likewise, the indole N10 is positioned in the minor groove between the 2-carbonyl oxygens of the two cytosines in the intercalation pocket, enhancing the overall stability of the complex. To accommodate the drug, the base pairs at the intercalation cavity are significantly separated by 7 Å, exhibiting a local helical twist of only 24° and thus being unwound by 12° with respect to canonical B-DNA.

The impact of basic side chains introduced at the cryptolepine C11 position was recently assessed in structure-activity relationship (SAR) studies [34]. Binding of a diverse set of N-(aminoalkyl)-cryptolepine-11-amine analogs to the dodecamer duplex d(GATCCTAGGATC) in a buffer at pH 7.4 was evaluated by fluorescence and absorption spectroscopy. A Job plot analysis of cryptolepine and one of its derivatives showed a 2:1 stoichiometry in the complexes, fully consistent with exclusive intercalation between the two available CC$\times$GG binding sites of the duplex in line with the crystal structure of cryptolepine-d(CCATGG)$_2$ (vide supra). As has been reported for cryptolepine and frequently found for DNA binding ligands, a second set of weaker binding sites was also apparent in the spectroscopic data for some of these analogs. Analysis of titration data demonstrated significantly enhanced binding affinities of the 11-substituted derivatives when compared with the parent cryptolepine. Association constants varied considerably with the chemical nature of the side chain and increased by more than one order of magnitude from $2.5 \times 10^5$ M$^{-1}$ for cryptolepine to $9 \times 10^6$ M$^{-1}$ for derivatives 1a and 1b with -NH(CH$_2$)$_3$NEt$_2$ substituents (Figure 5).
Selected analogs employed for these studies are ordered according to their duplex binding ability in Figure 5. Although a distinct structure-affinity correlation is not always obvious for the whole series, general trends emerge from the examples shown. Thus, N-aminopropyl and N-aminobutyl substituents are more beneficial compared with amines with a shorter C2-linker or a cyclic piperidine, suggesting insufficient flexibility of the latter substituents for optimizing additional interactions. Also, sterically more demanding branched alkyl chains seem to deteriorate binding in most cases. Interestingly, substituents with a terminal primary amino function are noticeably disfavored compared with secondary or tertiary amines on complex formation. The basicity for some of the amines has been found to decrease with shorter alkyl linkers associated with their greater proximity to the electron-withdrawing indoloquinoline structure and also from primary to tertiary amines in line with known hydration effects of protonated amines in water [29]. However, except for the anilino derivative 1k that lacks an aliphatic amine functionality, all 11-substituents shown in Figure 5 are expected to be mostly charged through protonation under physiological conditions and electrostatic interactions between the basic side chain and the anionic sugar-phosphate backbone should contribute to ligand binding for all analogs. In fact, the presence of such electrostatic forces for the aminoalkyl-substituted derivatives was demonstrated in the same study by a considerable weakening of interactions upon increasing the ionic strength of the solution [34]. Whereas variations in the small population of unprotonated amine may be important for biological activities owing to altered membrane permeabilities in the absence of transporter systems, the poorer binding on cell-free DNA by primary amine derivatives rather points to significant entropic contributions to binding from hydrophobic effects and/or from a larger energetic penalty for dehydration. Finally, whereas a single additional electron-withdrawing chloro substituent at position 3 was shown to be beneficial for the duplex affinity of two cryptolepine analogs with basic C11 side chains, affinity decreased again for a 3,8-dichloro-substituted derivative with its increased acidity at the pyrrolo NH.

Realizing the importance of N5-methylation for biological activities, fewer DNA binding studies have been reported on non-methylated quindoline derivatives. DNA binding of 11-carboxyphenyl-substituted benzo-δ-carbolines with two methoxy substituents at C4 and C9 and further derivatized at their carboxyl function through amide bond formation with di- and triamines was assessed with a GC-rich, AT-rich or randomly mixed non-self-complementary target duplex (Figure 6) [35]. Addition of 2a with its protonated dimethylaminoethyl side chain in a 5:1 ligand-to-duplex molar ratio had a negligible impact on the duplex thermal stability in a buffer with 100 mM NaCl and pH 7, only indicating weak binding at the most. However, employing a low salt buffer without any additional NaCl led to a small yet more noticeable increase of 2–3°C in the duplex melting temperature. Clearly, low ionic
strength conditions enhance favorable polyelectrolyte contributions because binding of a ligand with a charged side chain will release counterions from the duplex. In line with stronger electrostatic interactions between the nucleic acid and the doubly charged 2b derivative with two protonatable amino groups, a much larger thermal duplex stabilization with changes in melting temperatures of $\Delta T_m < 12\text{°C}$ was determined for 2b (F. Riechert-Krause, unpublished data). Obviously, less specific electrostatic interactions between charged side chains and the DNA sugar-phosphate backbone noticeably contribute to ligand binding affinity. At the same time, a clear sequence discrimination by the ligands was not apparent. Given their generally weak to moderate activity but at the same time reduced cytotoxicity compared with cryptolepine, as a result, neocryptolepine has been extensively studied and used as a lead compound for the development of antimalarial agents. Correlating with its lower cytotoxic effects, DNA interactions were found to be slightly weaker compared with cryptolepine [39–41]. However, neocryptolepine and its analogs were found to also intercalate into DNA preferentially at GC-rich sequences [40, 41]. Based on the crystal structure of the DNA-cryptolepine complex [32], the reduced affinity of neocryptolepine was suggested to result from the positioning of both nitrogens on the same side within the intercalation pocket, deteriorating stabilizing interactions [32].

In promoting interactions with a duplex, 11-carboxyphenyl-substituted 4,9-dimethyl-10H-indolo[3,2-b]quinoline 2c was covalently attached through amide bond formation to an aminoalkyl-derivatized oligonucleotide [36]. Duplex formation of the conjugate with a complementary oligonucleotide was studied at both pH 7 and pH 5 and yielded ligand-mediated thermal duplex stabilizations of $4\text{°C}$ and $11\text{°C}$ under neutral and acidic solution conditions, respectively. Because the duplex itself was not affected within the pH range examined, the significant gain in stabilization in the acidic medium suggests the presence of N5-protonated indoloquinolines with increased DNA binding affinity. In general, a positively charged indoloquinoline ring structure either through protonation or through N5-methylation as in the cryptolepine series seems to be a prerequisite of strong duplex binding.

An 11-carboxy-substituted quindoline derivative 2d lacking R₁ substituents as well as the unfused phenyl ring at C11 was also tethered through either an aminoalkyl, tetraethyleneglycol or threoninol linker to the 5′- or 3′-end of short oligonucleotides [37, 38]. Melting temperatures of duplexes formed with an unmodified and with the indoloquinoline-conjugated oligonucleotide were measured spectrophotometrically at pH 7. Depending on DNA sequence, attachment at either 5′- or 3′-terminus and on linker type, the tethered quindoline increased duplex melting temperatures by up to 8°C per interacting ligand. This was comparable to the stabilization by corresponding acridine derivatives known for their DNA intercalating ability and within the range found for the tethered phenyl-substituted derivative 2c (vide supra). A preference for CC sites as found for cryptolepine was not apparent and the strongest interactions were even observed for sequences with AT base pairs adjacent to the covalently attached ligand.

**Indolo[2,3-b]quinolines, indolo[3,2-c]quinolines and indolo[2,3-c]quinolines**

Neocryptolepine, the N5-methylated derivative of indolo[2,3-b]quinoline exhibits considerable biological activity but at the same time reduced cytotoxicity compared with cryptolepine. In SAR studies on various methyl-substituted indolo[2,3-b]quinolines [25] that were later extended to also include methoxy substitutions [42], binding affinities towards calf thymus DNA were determined (Figure 7). Similar to the results on indolo[3,2-b]quinolines, cytotoxicities as well as DNA binding abilities of the benzo-α-carbolines were strongly dependent on N5-methylation. Whereas neocryptolepines 4 showed significant activity and DNA binding affinity, no derivative of the 6H-indoloquinoline series 3 without N5-methylation exhibited noticeable activity or strong DNA binding. Given

![Figure 6](image-url)

**Figure 6** Substituted quinoline derivatives 2a–d.
the reported pK\textsubscript{a} values of 7.2–7.7 for the N5-methylated analogs (see also Table 1), neocryptolepines 4 should be N6-protonated to a large extent under physiological conditions. By contrast, the 6H-indolo[2,3-b]quinolines 3 with pK\textsubscript{a} values for the pyrrolo nitrogen protonation determined to be 5.6–6.0 should be mostly uncharged at neutral pH. The largest binding constants associated with high antimicrobial and cytotoxic activity were obtained for derivatives bearing the highest number of methyl or methoxy substituents on the pyrrolo side chain. Thus, whereas UV denaturation data were acquired at pH 7, 

Also, from the data of 5,11-dimethyl-indolo[2,3-b]quinoline 4a used as an internal reference and showing comparable binding affinity to the 5 derivatives, weaker binding of all aminoalkyl-functionalyzed derivatives 5 when compared with 4b and 4c can be concluded. Notwithstanding more specific steric and/or electronic effects, protonation at the basic side chain should promote DNA binding of the indoloquinoline through additional electrostatic interactions. Apparently, however, this additional contribution to binding cannot balance the lower affinity of a mostly unprotonated and thus neutral 6H-indoloquinoline ring system. One may also at least partially ascribe the differential DNA binding affinities found for the N5-methylated indoloquinoline derivatives 4 to the influence of the substituents on the pK\textsubscript{a} at the pyrrolo nitrogen. On closer inspection, there is a reasonable correlation between increasing binding affinity and increasing pK\textsubscript{a} values for the various methyl- and methoxy-substituted analogs. Thus, whereas low affinities are associated with derivatives exhibiting low pK\textsubscript{a} values, the two derivatives with the highest affinity 4b and 4c show the highest pK\textsubscript{a} within their series and thus feature the highest population of N6-protonated indoloquinolines that carry a positive charge. The reservation must be made, however, that titration experiments for determining association constants were performed at pH 5, whereas UV denaturation data were acquired at pH 7.

From the studies outlined above, indoloquinoline binding to duplex DNA seems to be favored with electron-deficient ring systems, that is, with indoloquinolines carrying a positive charge. Consequently, the pyrrolo...
nitrogen in 5-methyl-5H-indolo[2,3-b]quinolines or the pyridino nitrogen in the case of 6H-indolo[2,3-b]quinolines should be protonated under physiological conditions. Studies on the SAR of neocryptolepine derivatives corroborate the notion that small substituents may significantly influence DNA binding through their impact on indoloquinoline pK_a values and thus on the extent of protonation. Although no detailed binding studies have been performed, methyl green displacement assays on various substituted 5-methyl-5H-indolo[2,3-b]quinolines indicated a weakening of DNA interactions by electron-withdrawing substituents such as halogens, nitro or cyano groups [41]. By contrast, the biological activity of less cytotoxic 6-methyl-6H-indolo[2,3-b]quinolines was improved when amino functionalities were introduced at C11 [44]. Clearly, amino groups para positioned to the pyrido nitrogen are expected to considerably increase its basicity through strong electron-donating resonance effects.

Derivatives based on indolo[3,2-c]quinolines and in particular indolo[2,3-c]quinolines have to date attracted less attention and only few DNA binding studies have been reported. Employing methyl green displacements, isocryptolepine and isoneocryptolepine were again found to interact with DNA most likely through an intercalative mode of binding, although with lower affinity compared with cryptolepine or neocryptolepine [39]. DNA binding was analyzed quantitatively on several 3-chloro-substituted indolo[3,2-c]quinoline derivatives 6 (Figure 8) [45]. The determination of association constants upon titration with calf thymus DNA was hampered by solubility problems for some of the compounds but yielded values for two analogs with 9-(N,N-dimethyl)aminomethyl substitutions of 3×10^6 M^-1< K_a <5×10^6 M^-1 at moderate ionic strength and pH 7.5.

**Binding of indoloquinolines to triplex DNA**

Although the biology of non-canonical DNA structures is not fully understood yet, there is increasing evidence for their engagement in critical metabolic processes. Thus, DNA sequences with mirror repeat symmetry are often found in promoters and exons and can rearrange to form an intramolecular triplex or H-DNA structure upon releasing superhelical strain [46]. Recent experimental findings suggest that H-DNA is a source of genetic instability and H-DNA forming sequences are mutagenic in mammalian cells [47]. By contrast, triple helix formation through the sequence-specific recognition of a double-helical DNA target by a third strand oligonucleotide (TFO) has been recognized as a potentially powerful tool for various medicinal, diagnostic or technical applications.

The TFO in triplexes binds in the major groove of double-helical DNA forming specific hydrogen bonds to purine bases on one of the two duplex strands (Figure 9). In general, two types of triple helices have been characterized depending on the orientation of the third strand. In the pyrimidine motif, a homopyrimidine strand binds to a homopurine sequence of the duplex DNA in a parallel orientation with the formation of isomorphous T×AT and C×GC triplets. Because cytosine bases in the third strand have to be protonated in order to form two Hoogsteen
hydrogen bonds to a guanine base, a low pH value is generally required for stable triplex formation. In a second triplex motif, a purine third strand binds in an antiparallel orientation with respect to the oligopurine tract of the underlying duplex forming G×GC and A×AT triplets through reverse Hoogsteen hydrogen bonds. In addition, triplex-forming oligonucleotides composed of T and G may also bind to a homopurine stretch of double-helical DNA with an orientation that depends on the particular base sequence (for a review see [48]). Ligands that bind with high affinity and selectivity to triplex DNA are of considerable interest for alleviating the often low triplex stability, for example, for trapping or inducing triple-helical structures under physiological conditions. Also, corresponding compounds in either their free form or covalently bound to a TFO may be useful as signal transducers in detection methods that are based on triplex methodology or as potential tools for modifying or damaging target sequences that are recognized by the triplex-forming third strand [49–51].

In 2003, dialysis competition assays and mass spectrometry experiments with a set of different nucleic acid structures demonstrated that triple-helical rather than double-helical DNA constitutes the preferred DNA target for natural cryptolepine and neocryptolepine alkaloids [19]. Whereas the affinity for the favored poly(dA)×2poly(dT) triplex was significantly higher for cryptolepine, neocryptolepine was shown to be more selective in the discrimination between triplex and other DNA structures. In a series of papers, the triplex interaction of an 11-phenylindolo[3,2-b]quinoline 2c (Figure 6) tethered to a TFO was assessed in detail by various spectroscopic as well as calorimetric techniques [36, 52–54]. The studies not only provided information on the sequence selectivity, pH dependence and thermodynamics of binding but also revealed structural details of the triplex formed by the TFO-indoloquinoline conjugate with a target duplex. Employing various triplex constructs and TFO conjugates with the indoloquinoline covalently attached to terminal as well as internal positions of the triplex-forming oligonucleotide, strong triplex-specific interactions were observed with the ligand intercalating either between two base triplets within the triplex stem or between the terminal base triad and an adjacent base pair at the triplex-duplex junction. The latter was found to be the favored binding site by providing the largest triplex thermal stabilization. The determination of thermodynamic profiles revealed that triplex binding of 2c when attached to the 5′-end of the TFO only adds a small amount of -0.7 kcal mol⁻¹ to the strongly exothermic triplex formation. By contrast, tethering the indoloquinoline to the TFO considerably reduced entropic penalties upon binding and even resulted in a favorable entropy change. In fact, the ligand free energy of binding comprising almost 20% of the total standard free energy of triplex formation mostly benefited from the positive entropy of drug binding [53].

The strength of binding was also shown to strongly depend on pH as expected for a ligand that is subject to different degrees of protonation within the pH range examined. In line with a higher triplex affinity for the protonated species, drug-mediated thermal triplex destabilizations increase with decreasing pH. Whereas a pK_a value of 4.5 was determined for the protonated pyrido nitrogen in the non-conjugated 2a analog, triplex binding of the ligand was associated with a large increase of its apparent pK_a value by more than two pH units [53]. Consequently, the extent of 2c protonation will considerably vary with pH under slightly acidic and neutral conditions.

Drug protonation associated with a positive charge on the polyaromatic ring system was also found to affect the sequence selectivity of triplex binding. With 2c tethered at the 5′-end of the TFO, the most effective triplex stabilization was observed with a terminal TAT triplet located at the 5′-junction under low pH conditions [54]. A significant drop in triplex stabilization in the case of a CGC triplet at the junction was attributed to unfavorable charge repulsions between a partially protonated drug and a protonated third strand cytosine at the intercalation site. Intercalative drug binding at the triplex-duplex junction for the indoloquinoline conjugate was confirmed by NMR structural studies on a specifically isotope-labeled triple helix [54]. Based on NOE data, two well-defined molecular models for coexisting minor and major complexes could be constructed (Figure 10). The two species differ by a 180° rotation of the indoloquinoline within the intercalation pocket. The 11-phenyl substituent is oriented almost perpendicular to the plane of the indoloquinoline in the major groove. Restricted by the covalent attachment to the TFO, the pyrido nitrogen points towards the minor groove, contrasting with the crystal structure of the duplex-intercalated cryptolepine (Figure 4). Interestingly, both complexes exhibit significant stacking interactions with the two adjacent Watson-Crick paired bases but only poor additional stacking with the Hoogsteen-bound third strand thymine base of the terminal TAT triplet (Figure 10). Apparently, preferential triplex binding of these indoloquinolines is not primarily driven by more extensive stacking interactions with the base triads of the triplex but rather indicates other contributions to be more significant for the discrimination of secondary structure. Also, keeping extensive π-π stacking with the base pairs, the specific orientation of the indoloquinoline ring system...
Studies on the triplex formation with a TFO-indoloquinoline conjugate were also reported for the 11-carboxy-substituted quindoline derivative (Figure 6). Here, no or only a very weak stabilization of formed parallel triplexes was observed at pH 6. Notwithstanding different triplexes and buffer conditions used for the measurements, the electron-withdrawing 11-carboxy substituent in is expected to significantly lower the basicity at the N5 nitrogen, and even under slightly acidic conditions only a very small amount of protonated ligand with its higher affinity may be present in this case.

Very recently, the thermodynamics of triplex binding of free 2a and 2b carrying an alkylamine side chain was also evaluated in more detail [55]. UV melting experiments with parallel triplexes indicated a strong preference for TAT-rich triplexes in line with intercalation between TAT base triads and unfavorable electrostatic interactions with neighboring third strand cytosines. The basic substituents of 2a and 2b provide additional electrostatic contributions to binding. Consequently, binding affinities increase for 2b with its two protonable side chain amines and reach a triplex thermal stabilization of up to 22°C in a 100-mM NaCl containing buffer at pH 6. At the same time, the preference of the indoloquinoline ligand to bind triplexes with a longer run of TAT triplets was noticeably promoted by the longer diamine substituent. In addition to mostly unspecific electrostatic interactions, longer polymamines are suggested to progressively also interact through more specific contacts within one of the triplex grooves. Especially when positioned in the Watson-Hoogsteen groove, a more positive electrostatic potential from protonated cytosines will impair interactions with a positively charged side chain.

Compared with parallel triplexes with pyrimidine third strands, an antiparallel triplex with a GT-containing TFO was found to constitute an even better target for the 11-phenyl-substituted indoloquinolines [55]. Calorimetric measurements showed a significantly exothermic binding process with an enthalpy of binding that becomes increasingly favorable at lower pH. Although a positive entropic term also contributes to the free energy of binding in this case, binding of the unconjugated indoloquinoline is enthalpy-driven and thus contrasts with the binding of the TFO-indoloquinoline conjugate.

Unfortunately, a comparison of binding data reported for different ligands in the past is hampered by the strong influence of particular experimental conditions on the binding process. Thus, the concentration, molar ratio, ionic strength, buffer pH and target sequence all contribute to the determined binding affinities. To directly compare the triplex binding ability of 2a with natural cryptolepine, we
measured UV melting temperatures and ligand-mediated thermal stabilizations $\Delta T_m$ of an intramolecular TAT-rich pyrimidine triplex construct under standardized experimental conditions. Also included for an evaluation of relative triplex affinities was another synthetic analog 11-(N,N-dimethyl)aminopropyl-indolo[3,2-b]quinoline-11-amine, originally developed as a quadruplex binding ligand (vide infra) and termed SYUIQ-5 (Figure 11). Changes in triplex-duplex transition temperatures are given in Table 2 for three different pH values, highlighting the impact of protonation and charge on the binding affinity.

Whereas triplex stabilization through 2a becomes smaller with increasing pH due to a decreasing population of protonated drug (vide supra), both cryptolepine and SYUIQ-5 exhibit a noticeably enhanced thermal stabilization towards higher pH values. It should be noted that the thermal stability of the pyrimidine triplex itself strongly depends on pH and decreases from 38.1°C to 12.6°C when going from pH 5.5 to pH 7. Any ligand with exothermic protonation equilibria for cryptolepine and SYUIQ-5 have been found to not significantly affect triplex stabilities at pH 6.2 [57]. Given the considerable triplex affinity of indolo[3,2-b]quinolines with their BfPI-like geometry, differential triplex binding as found for isomeric benzopyrroloindoles should not primarily originate from their specific ring structure as a critical determinant of stacking interactions. Also, pH-dependent absorbance and fluorescence measurements suggest that all isomeric benzopyrroloindoles are fully protonated at the pyrido nitrogen at pH≤7 [58], questioning various degrees of protonation as a major source for the different triplex affinities. Therefore, the aminoalkyl side chain may again play a major role in the binding characteristics of BePI, BgPI and BfPI. In fact, molecular modeling studies indicated that differently oriented side chains on the intercalated ligand occupy different grooves of the triplex and their accommodation severely compromises stacking interactions in the case of the BfPI polyaromatic structure [57].

In an attempt to further extend the size of the aromatic ring system in BePI and BgPI for improved stacking

**Figure 11** 11-Substituted indolo[3,2-b]quinoline SYUIQ-5.

**Table 2** Changes in triplex thermal melting $\Delta T_m^{2a}$ upon addition of indoloquinoine ligands.$^a$

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\Delta T_m^{2a}$, pH 5.5</th>
<th>$\Delta T_m^{2a}$, pH 6.0</th>
<th>$\Delta T_m^{2a}$, pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>15.2±0.9</td>
<td>12.3±0.4</td>
<td>10.3±0.7</td>
</tr>
<tr>
<td>SYUIQ-5</td>
<td>25.8±0.6</td>
<td>29.9±0.6</td>
<td>38.8±0.6</td>
</tr>
<tr>
<td>Cryptolepine</td>
<td>12.4±0.6</td>
<td>15.9±0.5</td>
<td>20.3±0.5</td>
</tr>
</tbody>
</table>

$^a$In 20 mM Na cacodylate, 100 mM NaCl, pH 5.5/6.0/7.0.
interactions with all three bases of a base triplet, several 13H-benzo[4,5]indolo[3,2-c]quinolines (B[4,5]IQ, 7a–c) and 13H-benzo[6,7]indolo[3,2-c]quinolines (B[6,7]IQ, 8a–g) were synthesized and tested for their triplex-specific binding through UV melting experiments (Figure 13) [59, 60]. Generally, these pentacyclic benzo-fused indoloquinolines showed a remarkable DNA triplex thermal stabilization when bound to a preferred TAT-rich triple-helical oligonucleotide. As suggested by molecular models, stabilization was lower in all cases for the B[4,5]IQ derivatives as a result of reduced stacking interactions. No difference in binding was observed for positional isomers 8a and 8d with methoxy substituents either at the 10- or 11-position. Although more noticeable, differences were only modest among analogs 7a–c and 8a–c, differing in the length and N-substitution of their aminoalkyl side chain. However, whereas the two amino-substituted B[6,7]IQ isomers 8f and 8g were the least effective ligands with a ΔTm of approximately 40°C, an unprecedented triplex thermal stabilization of ΔTm = 65°C was observed at pH 6.2 for the B[6,7]IQ derivative 8e carrying an electron-withdrawing nitro substituent at C11. It should be mentioned, however, that these compounds also exhibit significant binding to double-helical DNA and thus possess only moderate selectivity in many cases.

**Binding of indoloquinolines to G-quadruplexes**

G-Rich DNA sequences with the ability to fold into four-stranded structures termed G-quadruplexes have attracted deep interest in recent years due to their widespread occurrence in eukaryotic chromosomes and their potential regulatory role in vivo. G-Rich tandem repeats prone to folding into quadruplex structures are found at the single-stranded 3′-terminus of telomeres which protect the chromosome ends from recombination, end-to-end fusion and degradation. The formation of quadruplex structures was shown to interfere with telomerase, an enzyme responsible for telomere maintenance in tumor cells. Thus, ligands inducing or stabilizing quadruplexes are effective telomerase inhibitors and may be used as anticancer agents. Other G-rich sequences appropriate for quadruplex formation are highly prevalent in the promoter region of several human genes and oncogenes such as c-myc. The human c-myc gene is involved in cell proliferation, apoptosis and senescence and its overexpression may lead to a variety of malignant tumors. Finally, oligonucleotide aptamers with quadruplex-forming sequences such as the thrombin binding aptamer may selectively act as inhibitors of signal transduction or transcription through the recognition of a particular target.

Recently, much effort has been devoted to anticancer therapeutic strategies that are based on the development of quadruplex binding ligands (for a review, see [61] and [62]). Intramolecularly folded quadruplexes comprise a core of at least two stacked G-tetrads connected by interwining mixed sequences in various folding topologies (for a review, see [63]). The G-tetrad itself consists of four guanine bases associated within a common plane through a cyclic array of hydrogen bonds (Figure 14). The four 6-carbonyl oxygens of such a planar G-tetrad arrangement are oriented towards a central cavity with a negative electrostatic potential, forming a specific binding site for monovalent metal ions. The stacked G-tetrads form a right-handed helix and the four backbone segments with attached contiguous guanine bases may be in either a parallel or an antiparallel mutual orientation depending on the specific topology. As a result, glycosidic torsion angles and the width of grooves formed between the DNA strands may vary considerably [61]. These grooves with their distinct geometry together with loop regions have been recognized in addition to the G-tetrad planes as potential interaction sites with small molecules.

One of the first indoloquinolines tested for quadruplex binding were the methoxy- and nitro-substituted pentacyclic benzindoloquinoline (BIQ) derivatives 8a, 8d and 8e (Figure 13) originally developed as triplex intercalators [64]. Binding to a quadruplex formed by the human telomeric motif was confirmed through changes in the absorbance and fluorescence of the BIQs upon DNA titration. Changes in quadruplex melting temperatures due to

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**Figure 13** Structure of 13H-benzo[4,5]- and 13H-benzo[6,7] indolo[3,2-c]quinoline derivatives.
ligand binding were monitored using a fluorescence resonance energy transfer (FRET) methodology and yielded a ligand-mediated $\Delta T_m$ of approximately 10°C under the solution conditions employed. However, an analog of 8d with a shorter aminoethyl side chain substituted for the aminopropylamino side chain at C6 was less effective in quadruplex stabilization. A good correlation was found between quadruplex thermal stabilization and telomerase inhibition, measured by a telomerase repeat amplification protocol (TRAP) assay. Although a competitive equilibrium dialysis showed that BIQs preferentially interact with DNA quadruplex structures, considerable binding was again observed for double-helical DNA, compromising specificity towards telomerase inhibition.

A search for efficient quadruplex binding ligands based on the indoloquinoline scaffold has only started at the beginning of the 21st century. In line with the external stacking on the terminal G-tetrad as found for several quadruplex binders with a large planar aromatic surface [62], indoloquinolines are also expected to provide strong interactions when stacked on an outer G-quartet. Notwithstanding benzoannelated BIQs mentioned above, indoloquinoline ligands developed for quadruplex recognition are largely derived from quindoline or its N-methyl derivative cryptolepine. Because the latter two compounds lack sufficient activity and/or selectivity, efforts have been directed towards the synthesis of derivatives carrying different substituents at various positions to optimize stacking energies and in particular to add more specific interactions between side chains and the grooves or loop domains of the quadruplex target.

A quindoline derivative 9 with two basic N,N-dimethylaminoethyl substituents at C2 and N10 was the first 10H-indolo[3,2-b]quinoxaline specifically designed as a telomerase inhibitor with the ability to induce and stabilize quadruplex formation of single-stranded telomere sequences (Figure 15) [65]. Although no quadruplex binding studies of 9 were reported, its inhibitory activity against telomerase suggested its stabilizing effect on quadruplex structures. However, inhibition was only moderate with $IC_{50}$ in the lower micromolar range as evaluated in a modified TRAP assay. In fact, computer models of 9 bound to the human telomeric G-quadruplex structure indicated that the two aminoethyl side chains may be of insufficient length for more favorable interactions.

In further optimizing quindoline derivatives for quadruplex recognition, reduction of 10a with LiAlH₄ yielded the more potent analog 12a with its 2,7-di-aminoalkyl substitution pattern and a longer propyl linker (Figure 15) [66]. Here, molecular models indicated stable interactions between basic side chains and DNA backbone. Employing a FRET-based DNA melting assay, the relative ability for quadruplex stabilization was determined for 12a, 12b, the N10-acetylated precursor 10a with its less basic amide side chains and the monoalkylated analog 11a. Whereas a pyrrolidine moiety in the side chain was slightly favored over a piperidine with respect to quadruplex affinity, the loss of one aminoalkyl substituent in 11a considerably deteriorated quadruplex stabilization. No noticeable stabilization was detected for 10a. In further expanding the series of 2,7-dialkylated quindolines, 12c–g were prepared more recently [67]. Among the derivatives with phenyl-containing appendages, 12d and 12e showed the best quadruplex stabilizing effects coupled with promising selectivity for quadruplex over duplex DNA.

In another approach to design effective quadruplex stabilizing indoloquinolines, an N-alkylamino substituent was introduced at the C11 position as a key structural element for compounds 13 (Figure 16) [26, 68]. The amino substituent significantly increases electron density and
thus basicity at the pyrido nitrogen. Based on the corresponding \( pK_a \) values determined to be approximately 8.3 for compounds 13, all derivatives should be mostly protonated at N5 under physiological conditions. Binding affinities of the quindoline analogs 13 towards the human telomeric DNA sequence \( d[G_3(T,GAG)_3] \) were evaluated in the absence of metal ions through temperature-dependent changes in CD spectra. The \( \Delta T_m \) values determined in a 10-mM Tris-HCl buffer at pH 7.4 after ligand addition to the quadruplex range from 14.0°C for 13e with a terminal hydroxy group up to 22.3°C for 13b with a tertiary amino group in the side chain. A competition dialysis experiment also showed that 13a favored quadruplex over duplex binding. The quindoline derivatives not only stabilized the G-quadruplex structure but were also able to induce folding of the telomeric sequence into a quadruplex. Given the typical CD spectral signatures for the differently folded G-quadruplexes, it was concluded that the indoloquinolines 13 may suppress structures with parallel strand arrangements.

The same series of quindoline derivatives was later also tested for its quadruplex-stabilizing ability on a 27mer G-rich sequence Pu27 that functions as a regulatory element in the human c-myc oncogene promoter [69]. The Pu27 oligomer is known to form parallel G-quadruplexes in solution containing potassium ions. Results from temperature-dependent CD spectroscopic measurements and competition dialysis assays on the binding of 13b-f to Pu27 can be summarized as follows: (i) side chains with alcoholic hydroxy groups showed a lower quadruplex thermal stabilization compared with side chains with a more basic terminal amino group; among the latter no significant differences in \( \Delta T_m \) were observed; and (ii) quadruplexes are favored for binding over duplex and triplex structures with a higher selectivity found for 13f compared with 13e with its non-protonated substituent. Interestingly, unlike analogs with a hydroxy group derivatives with a terminal amino group in their side chain seemed to favor loop isomers with double-nucleotide loops over isomers with single-nucleotide loops in the absence of K+. Such loop isomeric structures of the Pu27 quadruplex have previously been found to coexist in a dynamic mixture and molecular modeling studies suggest that electrostatic and hydrogen bond interactions between the side chain and a phosphodiester within the loop may be responsible for this particular selectivity.

In a mechanistic study on the inhibition of cell proliferation by the quindoline derivative 13c (SYUIQ-5), relative binding affinities for a telomeric G-rich DNA and Pu27 were assessed in more detail [70]. FRET-based DNA
melting experiments and isothermal titration calorimetry (ITC) measurements indicated that 13c favors binding to the parallel P27 c-myc quadruplex over the telomeric sequence, expected to fold into a hybrid-type G-quadruplex in the presence of potassium ions. Corresponding ITC titrations yielded binding constants for the ligand in a K\(^+\)-containing buffer of 4 \times 10\(^4\) and 1.8 \times 10\(^3\) M\(^{-1}\) for the c-myc and telomeric G-quadruplex target, respectively.

In following the concept of introducing charges and electron deficiencies to the indoloquinoline ring system for more favorable interactions with DNA, quinoline derivatives 13 were additionally N\(_5\)-methylated to yield cryptolepine analogs with a stable positive charge on the aromatic core (Figure 17) [71]. Indeed, a comparison of FRET-derived melting temperatures of the human telomeric quadruplex upon binding 13c and its N\(_5\)-methylated analog 14b revealed that introduction of the positive charge by methylation significantly improved the ligand-mediated stabilization of the quadruplex structure. Also, upon addition of 14b to the telomeric sequence in K\(^+\)-containing buffer, the CD spectra changed and observed CD signatures pointed to a shift from a hybrid-type G-quadruplex. Among the series of compounds 14, the largest stabilization with a \(\Delta T_m\)\(^{-18}C\) in 60 mM potassium buffer was observed for analogs 14b and 14d with an aminopropyl side chain. By contrast, the lowest stabilization with a \(\Delta T_m\)\(^{-8}C\) was determined for analogs 14g and 14h with a hydroxyl group replacing the basic amino group at the side chain terminus. Thermal stabilizations also decreased, although more moderately, with a shortening of the propyl to an ethyl linker and with the introduction of a terminal morpholino residue. To also test the impact of additional electron-withdrawing substituents, one or two fluorine atoms were introduced at the 7- and 9-position of 14a–d. However, an additional reduction of electron density as expected in the presence of the fluorine substituent did not result in stronger interactions with the electron-rich π-system of the G-quartets.

Three other closely related analogs 15a–c were screened for their G-quadruplex stabilization and associated anticancer profile (Figure 18) [72]. FRET melting assays on a human telomeric G-quadruplex demonstrated that 15a and 15b with aliphatic amine side chains both constitute quadruplex ligands with high affinity, leading to thermal stabilizations \(\Delta T_m\) of \(\sim\)20°C. By contrast, 15c comprising an N-substituted aniline moiety in its side chain exhibits a drastically decreased G-quadruplex stabilization with a \(\Delta T_m\) of only 4°C, attributable to its less basic aromatic amine functionality. Interestingly, however, the selectivity for quadruplex against duplex targets was increased in the case of 15c when compared with 15a and 15b with their much higher quadruplex binding affinity.

A comparison of corresponding melting data for 15a, 15b as well as of 14d also revealed that there is no noticeable impact on quadruplex binding when replacing the N-aminobutyl side chain in the cryptolepine-11-amine by a less flexible N-piperidyl or by a shorter N-aminopropyl residue.

Recently, a series of 11-piperazinyl-substituted quinodine analogs were synthesized as potential c-myc G-quadruplex stabilizing compounds and tested for their impact on c-myc gene expression [73]. Representatives of the various mono- and disubstituted derivatives 16–19 are shown in Figure 19. The thermal stabilization of a c-myc quadruplex as a measure of binding affinity was evaluated by temperature-dependent ellipticities. For the monosubstituted compounds 16a–d, stabilization follows the order 16d<16a<16b<16c, that is, a basic amino group in the side chain R improves binding and the three-carbon linker in 16c is favored over a two-carbon linker in 16b for quadruplex binding. Attaching a second N,N-dimethylaminoalkyl substituent at the indole nitrogen of 16b results in a further enhanced thermal stabilization (\(\Delta T_m\)=16.5°C for 17b compared with \(\Delta T_m\)=6.7°C for 16b). However, this apparent cooperativity effect with the double substitutions requires two basic amine substituents and completely vanishes for 17a with a methyl substituent at the piperazine moiety (\(\Delta T_m\)=2.8°C). Also, a second substituent at C2 in derivatives 18 only seems

![Figure 17](https://example.com/figure17.png)

**Figure 17**  Structure of N5-methyl quindoline-11-amine derivatives 14a–h.

![Figure 18](https://example.com/figure18.png)

**Figure 18**  Structure of N5-methyl quindoline-11-amine derivatives 15a–c.
to significantly improve binding if basic and thus protonated as in 18b. The impact of basicity and thus protonation at the indoloquinoline N5 was again demonstrated by the low binding affinity of analog 19 with the 11-piperazinyl substituent replaced by a substituted phenyl residue.

In contrast to thermal melting studies, an evaluation of binding through competition dialysis revealed that compound 16b binds with higher affinity to G-quadruplexes than derivative 17b while exhibiting only poor selectivity for different G-quadruplexes. It should be mentioned that G-quadruplex affinities for 16b and 17b showed only a poor correlation with their intracellular transcriptional regulation. Also, the anticancer activity of both analogs was found to be mediated through potential interaction on different genes rather than to the targeting of the single c-myc quadruplex as originally anticipated.

In 2011, a NMR solution structure was reported for a c-myc G-quadruplex with two bound 13f ligands [74]. To only allow for the formation of the major loop isomer in a K+ containing solution, the 22mer G-rich oligonucleotide Pu22 of the c-myc promoter region was double-mutated by two G-to-T substitutions (Figure 20). Also, decreasing the salt concentration to 10 mM K+ improved the binding of 13f and enabled a complete NMR structure determination at pH 6.

Similar to free Pu22, the ligand bound c-myc sequence adopts a parallel quadruplex with one TA and two single-nucleotide T reversal loops. Two drug molecules stack on the external guanine tetrads at each end (Figure 21). However, flanking sequences were found to have a considerable impact on the strength and mode of binding. The 5′-TGA and TAA-3′ terminal segments were significantly rearranged upon drug binding, reminiscent of ligand-induced conformational changes in riboswitches. Thus, adenine at position -1 and the indoloquinoline ligand form another stack above the external G-quartet and the guanine base at position -2 is recruited to cap this additional plane. The indoloquinoline core shows stacking interactions with two guanine bases of the G-quartet below and A(-1) stacks above a third guanine of the tetrad. A similar configuration is found for 13f binding at the 3′-external G-tetrad. Here it is the thymine base at position +1 that spans a common plane with the ligand above the G-quartet. Again, the adjacent adenine +2 stacks over this additional plane and forms a drug-induced binding pocket. Although similar in geometry, the two binding arrangements at the 3′- and 5′-faces were suggested to be stabilized in different ways. Whereas ligand binding at the 3′-end may strongly benefit from a potential hydrogen bond between NH5 of the ring-protonated indoloquinoline and the carbonyl O4 of T(+1), there is no such hydrogen bond interaction to A(-1) in the opposite binding pocket. Instead, stacking interactions also involving G(-2) seem to be more important for 5′ binding, with its more accessible and hydrophobic face. This is corroborated by the observation that a base substitution at position -2 may significantly weaken drug binding. By contrast, a decreasing ionic strength improves binding at the 3′-face as expected for hydrogen
bond interactions. Overall, the three-dimensional structure illuminates the critical impact of flanking bases in c-myc quadruplexes on the affinity as well as specificity of ligand binding.

Concluding remarks

The significant biological activity of many natural as well as synthetic indoloquinoline derivatives seems to mostly derive from their interaction with genomic DNA. However, alternative DNA structures have also been found to constitute high-affinity targets, making the tetracyclic indoloquinoline scaffold with its strong intercalating ability an attractive lead structure in the development of DNA binding ligands for various pharmaceutical as well as non-pharmaceutical applications. An electron-deficient, positively charged indoloquinoline core has in many cases been shown to improve stacking interactions not only with base pairs of a Watson-Crick duplex but also with base triads or G-tetrads in triplexes and quadruplexes. Consequently, the introduction of proper substituents at the aromatic skeleton to trigger protonation at the pyrido nitrogen by increasing its basicity is a practical way of enhancing DNA binding affinities. Alternatively, a stable positive charge may be introduced by N5-alkylation with the quaternization of the aromatic ring nitrogen. As an added benefit, N5-protonated or N5-alkylated cationic derivatives should exhibit increased water solubility. However, when trying to further reduce the π-electron density of the aromatic core through electron-withdrawing substituents, the influence of the latter on the particular pKa values must be considered. Clearly, shifting protonation equilibria from a protonated towards a deprotonated neutral species will in most cases be counterproductive and impedes a strong ligand binding.

In addition to the strength of binding, the binding selectivity constitutes a critical parameter for any potential application of indoloquinolines. A planar aromatic ligand should preferably match the geometry of a base pair, base triad or G-tetrad to maximize binding energies as a result...
of stacking interactions. Generally, this calls for a polyaromatic core of increasing size when going from a duplex to three- or four-stranded DNA targets. Indeed, several of the most potent quadruplex ligands follow such a design and are based on extended aromatic ring systems. By contrast, the tetracyclic indoloquinoline skeleton was found to only stack over two bases, limiting stacking interactions in triplexes and especially quadruplexes. Benzoannelated indoloquinolines with an extended pentacyclic ring system alleviate this problem and have been shown to exhibit more favorable triplex as well as quadruplex binding properties. Alternatively, the strength of binding can be considerably increased by proper side chains attached to the indoloquinoline core at suitable positions. Such appendages when positioned in a groove of the nucleic acid structure may add to the overall free energy of binding through additional enthalpic (electrostatic, H-bond, van der Waals) as well as entropic (desolvation) contributions.

A discrimination of duplex, triplex and quadruplex structures solely based on the different hydrogen-bonded nucleobase arrangements is not expected to allow for a sufficient specificity of the tetracyclic indoloquinoline ligands. Rather, the geometry of the various grooves formed by the DNA structures together with other structural elements such as junctions, loop regions or overhang sequences constitute critical targets of a more specific binding, that should involve side chains attached to the indoloquinoline skeleton. Because interactions within different DNA grooves or with more flexible loop regions are more difficult to predict, the rational design of substituted indoloquinoline derivatives tuned for a particular DNA target remains a challenge at present. However, with an ever increasing number of modified indoloquinolines combined with a better understanding of structure-affinity relationships, high selectivities in binding duplex, triplex or quadruplex structures but also in the recognition of structures with more subtle differences, for example, quadruplexes of different topology, may be possible in the near future.

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