# Design of Selective G-quadruplex Ligands as Potential Anticancer Agents

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**Abstract:** G-quadruplex structures are promising targets for design of cancer drugs with high selectivity and low toxicity. This review provides an update of the progress made over the last few years in the design of selective G-quadruplex ligands, and a comprehensive summary of the major design strategies and structural characteristics.

**Key Words:** G-quadruplex, G-quartet, ligands, substituted derivatives, affinity and selectivity, DNA interactions, telomerase inhibition.

## 1. INTRODUCTION

DNA is a classical and also an effective target for designing anticancer agents. The alkylating agents and topo poisons such as cisplatin, doxorubicin and topotecan which have been used for many years are still widely used in cancer therapy because of their proven efficacy. However, a major problem with the conventional chemotherapeutic agents is their strong toxicity and other side effects to the patients because of their poor selectivity, or nonspecific interactions with the duplex DNA. G-quadruplexes refer to the four-stranded structures formed by the guanine-rich DNA sequences which are most probably present in the telomeres of eukaryotic chromosomes. Telomeres have a crucial function in restricting the proliferative capacity of normal human cells but most tumor cells have acquired telomerase activity to maintain their unlimited growth potential. To improve the selectivity and reduce the side effects of DNA-interactive drugs, many researchers have focused on designing molecules targeting at G-quadruplexes (G4) [1].

The formation of G4 structures by intermittent runs of guanines in nucleic acids was first proposed by Davies and colleagues in 1962 [2], only a few years after Watson and Crick had proposed the DNA double-helix in 1953. Potential G-quadruplex structures have been identified in telomeric DNA sequences and also more recently in non-telomeric genomic DNA promoters [3,4]. These higher-order DNA structures represent a new class of molecular targets for selective DNA-interactive compounds in view of the high telomerase activity and abnormal overexpression of oncogenes in most cancer cells but not in normal cells [1]. These compounds are designed to inhibit the telomerase or to inactivate the transcription activity of oncogenes (e.g., *c-Myc*, *c-kit*, *Bcl-2*, etc.) [5-8]. The design of drugs targeting at the telomeres or promoter quadruplexes is a rational and promising approach.

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The design of selective G4-ligands was initially focused on differentiating between duplex and quadruplex DNA species, since the ligand interaction with duplex DNA leads to toxic and side effects on normal tissues. With an increasing number of quadruplexes identified in the genome, ligand design has also been directed at selectivity among different quadruplex species. This review is dedicated to the recent development in the search and design strategies for selective G-quadruplex binding ligands.

## 2. G4 STRUCTURES AND COMPLEXES WITH LIGANDS

### 2.1. G-Quartets and G-Quardruplexes

Apart from the primarily right-handed double helix (Fig. **1A**), DNA can adopt higher-order and functionally-useful structures, such as the G-quadruplexes (Fig. **1B**). The building blocks of G-quadruplexes are the G-quartets (or G-tetrads) which are derived from the association of four guanines into a cyclic Hoogsteen hydrogen-bonding arrangement with two hydrogen bonds between two neighboring guanine bases. The G-quartets stack up with one on top of another to form the G-quadruplex DNA structures.

G4 structures exhibit a high degree of polymorphism which depends on the characteristics of strand stoichiometry and loops, and the metal cations coordinated with the G-quadruplexes. G-quadruplexes can be formed by folding of a single guanine-rich sequence (intramolecular) or by the association of two or four separate strands (intermolecular). Polymorphism can also be created by the relative arrangement of strand polarity in various ways, all parallel, three parallel and one anti-parallel, adjacent parallel, or alternating parallel (Fig. **2**). Quadruplexes are designated as anti-parallel when at least one of the four strands is anti-parallel to the others, which is found in most of the bimolecular and many of the intramolecular quadruplex structures characterized to date. Meanwhile, the variations in strand polarity affect the appropriate location of the linkers, or loops, between G-rich segments formed either from a single-strand or from two strands. Thus, parallel G-strands require a connecting loop to link the bottom G-quartet with the top G-quartet, leading to propeller-type (double-chain-reversal) loops. Anti-parallel

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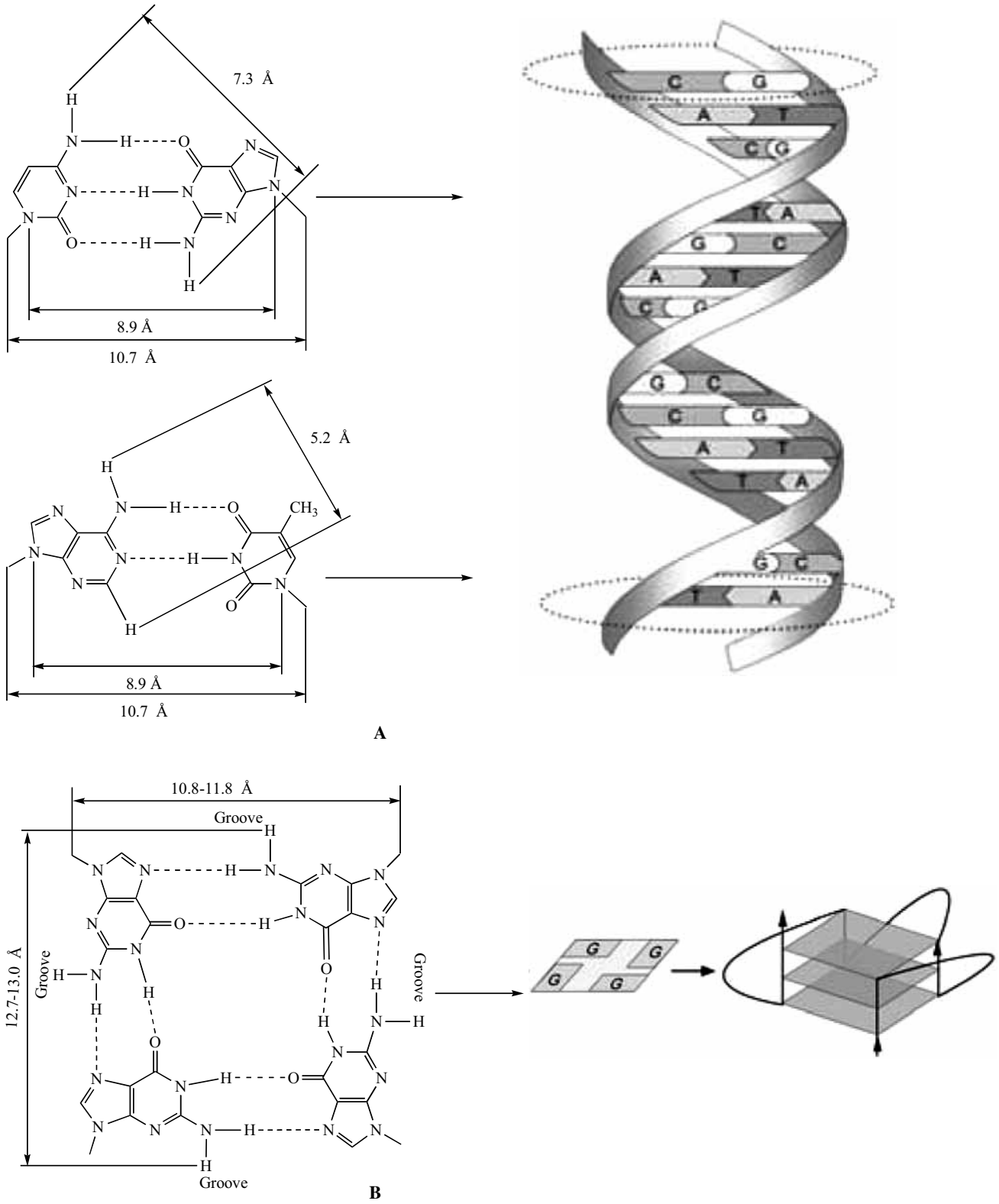



**Fig. (1).** Basic structures of B-type duplex DNA (A) and G-quadruplex (B).

G-strands can be linked either by lateral or diagonal loops, depending on whether the strands are adjacent or diagonally opposed (Fig. **2**). The sequence and size of loops usually have a dominant role in determining the quadruplex topology. Loop residues can themselves form stacking and hydrogen-bonding interactions, further stabilizing or destabilizing particular G-quadruplex folds [9-12].

Furthermore, G-quadruplexes are characterized by coordination of monovalent cations, usually $K^+$ and $Na^+$. The

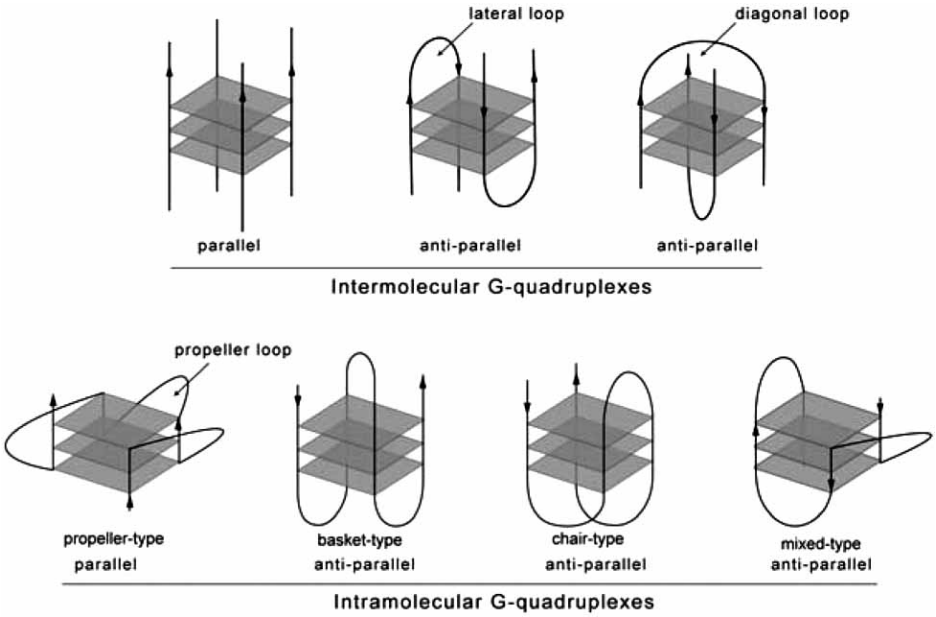



**Fig. (2).** Topology of G-quadruplex.

hole between G-quartets is well-suited for coordinating the right size of cations because the two planes of quartets are linked by eight carboxyl oxygen-6 atoms which have strong negative electrostatic potential to create a central channel in the G-quartet stack [13]. Variability of G4 topology from the same sequence can arise due to different characteristics of metal cation coordination in a G-quartet (Fig. **2**). For example, the human telomeric DNA sequence, d[AGGG $(TTAGGG)_3$] forms the anti-parallel topology with one diagonal and two lateral TTA loops in $Na^+$ solution, termed basket-type [14]. On the other hand, the potassium complex with this sequence has variable and complicated structures including anti-parallel basket- or chair-type structure, parallel propeller-type crystal structure [15-17], and mixed-type structure of one propeller and two lateral loops [18-20].

Because of the factors mentioned above, the parallel quadruplexes all have guanine glycosidic torsion angles in an anti conformation. On the other hand, G-quartets in anti-parallel quadruplexes are found in both *syn* and *anti* conformations, which are arranged into a specific topology and set of strand orientations [21]. Moreover, there are four grooves in quadruplex structures, defined as the cavities bounded by the phosphodiester backbones. Groove dimensions are variable, depending on the overall topology and the nature of loops.

### 2.2. G4-Ligand Complexes

In search for selective G4 ligands, it is imperative to understand their binding sites and schemes with the G-quadruplex. According to NMR studies [22], the G4 ligands usually stack over the terminal G-quartets on either end of the quadruplex, unlike the ligands intercalating into the base pairs or simply lying in the grooves of duplex DNA. Interestingly, distamycin, a non-coplanar DNA duplex minor groove binder, also stacks on the two ends of a G-quartet [23]. Moreover, the interaction of fluorinated polycyclic acridinium salt with the G-quadruplex is maintained by stacking two ligand molecules on the G-quartet core, and also by placing the positively-charged ligand above and below the ion channel at the center of G-quartet [24]. It is clear that G-quartet is the basic moiety for G4-ligand interactions which has a square aromatic surface of G-quartet much larger than that of the Watson-Crick base pairs (Fig. **1**). The strong and selective binding of the ligand molecule to the G-quadruplex may be attributed to its larger ring system, which overlaps completely with the four guanines in the G-quartet and allows the molecule to occupy the whole area of the quartet region. In addition, the core of G-quartet is much more electronegative which may be specifically attractive to compounds with a positive charge center.

The crystal structure of a parallel G4–daunomycin complex shows three daunomycin molecules stacking on the 5'-end of the G-quartet core, with their amine sugar moieties forming hydrogen-bonding and/or van der Waals interaction with the quadruplex grooves [25]. Similarly, the positive charges of TMPyP4 are in close contact with several negatively charged phosphates in c-*Myc* promoter as seen from the NMR solution structure of c-*Myc*-TMPyP4 complex, suggesting the contribution of electrostatic interactions to the stability of complex [26]. Meanwhile, the crystal structure of a complex of a bimolecular human telomeric G-quadruplex and the TMPyP4 molecule in the presence of $K^+$ ions reveals a parallel-stranded quadruplex with propeller loops that interact with two independent TMPyP4 molecules. The porphyrin molecules bind by stacking onto the TTA nucleotides, either as part of the external loop structure or at the 5' region of the stacked quadruplex, but no direct interaction with G-quartets [27]. In general, grooves, loops and their negatively charged phosphatic residues in G-quadruplexes are major sites for the design of drugs with high selectivity, especially for recognition of different G-quadruplexes. For example, G-quadruplexes with propeller loops obstruct ligands accessing the grooves, which are unfavorable for ligands with groove-recognition moieties, but can still accept edgewise and diagonal loops [22]. The loop feature of *c-kit* G-quadruplex is

the most important factor in designing its selective binders for other G-quadruplex structures [28].

## 3. METHODS FOR STUDYING SELECTIVE G4 LIGANDS

In the development and design of drug molecules targeting G-quadruplexes, effective assays are required for determining the selectivity of these compounds and studying their interactions with G4 structures. Fluorescence resonance energy transfer (FRET), competition dialysis and surface plasmon resonance (SPR) techniques have been most commonly used in screening selective G4 ligands.

FRET is a spectroscopic method that provides distance information of macromolecules in solution and is particularly useful to probe the secondary structure of guanine-rich sequences in which a donor and an acceptor are attached to one end of the oligonucleotide [29]. The destruction of quadruplex into single strand is often accompanied by the increase in distance between the donor and the acceptor, leading to a less efficient energy transfer from donor to acceptor. Thus, the melting temperature ($T_m$) of these fluorescent oligonucleotides can be detected in the presence of different molecules by RT-PCR to estimate the interaction between a ligand and the quadruplex. In addition, the same test may be performed in the presence of a large excess of other non-fluorescent DNA competitors (e.g., other G-quadruplexes, single strands and double strands), to screen out ligands that show preference for quadruplex over other structures.

Competition dialysis is commonly used to probe the binding selectivity of ligands to various nucleic acid structures based on equilibrium dialysis [30]. In this assay, the DNA-ligand solution is dialyzed through a membrane that allows only small ligands to pass through. When equilibrium is reached, the array of structures is in contact with free ligands at an equal concentration. The amount of ligands bound to each structure provides a direct measurement of the affinity for that structure, so that structural preferences can be readily compared.

The SPR method is useful for detecting the binding events at the surface of a thin metal film. It has found wide applications for determining the affinity and kinetics of drug–DNA interactions. Structural preference is determined based on the affinities of ligands with different DNA structures [31]. Other techniques, such as electrospray ionization mass spectrometry (ESI-MS) [32], competition polymerase stop assay [33], phage ELISA assay [34] and spectroscopic methods [35,36] have also been used to screen selective G4 ligands.

## 4. SELECTIVE G4 LIGANDS: FUSED AROMATIC SYSTEMS

### 4.1. Diamidoanthraquinones

Diamidoanthraquinones (**1**) (Fig. **3**) are one of the earliest and most widely characterized quadruplex-interactive ligands [37]. Structure-activity studies including cytotoxicity, binding property and telomerase inhibition have been performed for a wide range of diamidoanthraquinones. Some related compounds had previously been synthesized as conventional cytotoxic agents with affinity for duplex and triplex DNA-interactive compounds. Previous studies have found a significant correlation between *in vitro* cytotoxic potency and duplex-binding affinity of the diamidoanthraquinones, but no evidence for a significant correlation between G4 binding and telomerase inhibition activity [38-40]. Molecular modeling studies suggested that these compounds bind by a "threading intercalation mode" to G-quadruplex structures with two terminal amine side-chains protruding into grooves, analogous to their behavior with duplexes. Although two amido groups attached to the anthraquinone tricyclic system increased the effective length of the system from 7.5 to 12 Å, it was not sufficient for the G-quartet and four grooves [41]. As a result, diamidoanthraquinones are not desirable selective G-quadruplex agents.

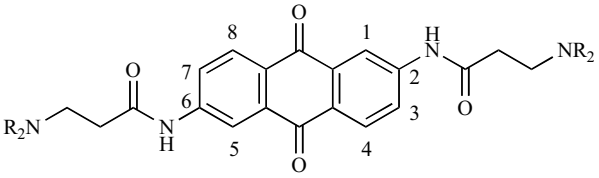
### 4.2. Substituted Acridines

A series of disubstituted acridines (**2**) (Fig. **3**) has been synthesized by the introduction of a positive charge in the centre of the chromophore to complement the channel of negative electrostatic potential that runs through the centre of a quadruplex. However, this approach led to little improvement of the affinity and selectivity in comparison with diamidoanthraquinones [42,43], due to the weak alkalinity of the nitrogen atom in the central ring. The incorporation of a third substituent, an anilino group, into the acridine chromophore at the 9-position (**3-12**) (Fig. **3**), which fits into a third groove in this model, enhanced the alkalinity of the nitrogen atom in the central ring [44].

According to SPR analysis, the disubstituted acridines had the similar binding constants with duplex and quadruplex, whereas the trisubstituted compounds had much stronger binding affinity to human quadruplex DNA (with 10–70 times larger binding constants) than to duplex [45,46]. However, the elongation of 3- and 6-side chains significantly decreased the quadruplex affinity for both groups of compounds (**3-6** and **7-12**). Of all the trisubstituted acridines, the 3,6-hexanamido derivative **12** displayed the highest quadruplex selectivity. This suggests that the added steric bulk of these ligands at the 3- and 6-positions is unfavorable for quadruplex or duplex binding. Conversely, **7** and **9** exhibited a stronger G4 binding affinity but a lower selectivity due to an increase in duplex binding. A balance between G4 binding affinity and selectivity against duplex binding should be taken into account.

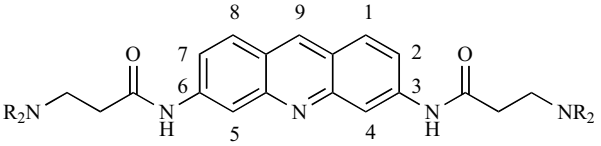
### 4.3. Trisubstituted Isoalloxazines

The design of isoalloxazines as potential G4 ligands was based on the finding that oxidized riboflavin binds to an intramolecular G-quartet with moderate binding affinity ($K_d$) of 1-5 μM [47]. Similar to the design of trisubstituted acridines, three amine substituents were introduced to the planar isoalloxazine scaffold (**13-18**) (Fig. **3**) for potential interactions with quadruplex loops, grooves and the negatively charged sugar-phosphate backbone [48]. None of these ligands showed significant binding to the duplex control in SPR assay or any detectable stabilization of duplex DNA in FRET analysis. The most remarkable was that compound **13** showed 14-fold G4 selectivity for *c-kit* over human telomeric sequence as discrimination between two different G-quadruplexes by a small molecule is a greater challenge than be-



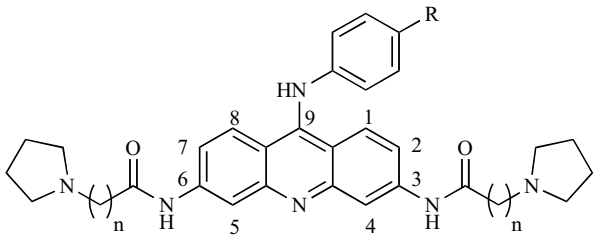
Diamidoanthraquinones

**1**

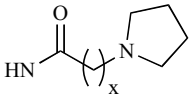


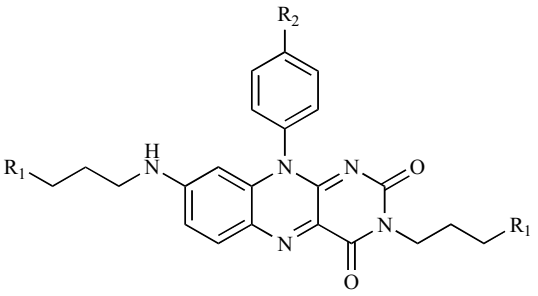
Disubstituted acridines

**2**



Trisubstituted acridines

| | | | |
|---|---|---|---|
| R = | $N(CH_3)_2$ | R = |  |
| **3** | n = 2 | **7** | n = 2 x = 1 |
| **4** | n = 3 | **8** | n = 2 x = 2 |
| **5** | n = 4 | **9** | n = 2 x = 3 |
| **6** | n = 5 | **10** | n = 3 x = 2 |
| | | **11** | n = 4 x = 2 |
| | | **12** | n = 5 x = 2 |



Trisubstituted isoalloxazines

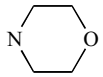
| NO. | $R_1$ | $R_2$ |
|---|---|---|
| **13** | $N(CH_3)_2$ | $N(CH_3)_2$ |
| **14** |  | $N(CH_3)_2$ |
| **15** |  | $N(CH_3)_2$ |
| **16** | $N(CH_3)_2$ | F |
| **17** |  |  |
| **18** |  | OMe |

**Fig. (3).** Structures of G-quadruplex ligands in tricyclic aromatic system.

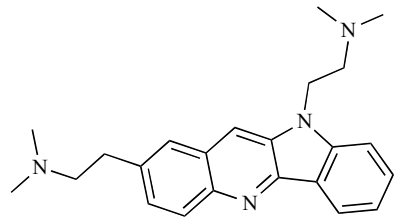
tween quadruplex and duplex. Although structural information of the ligand interactions is still limited, the results have shown that small coplanar tricyclic system with drug-like scaffolds has the potential to discriminate a specific G4 structure from duplex and other G-quadruplexes. The essential point is that appropriate number and configuration of substituent groups are introduced to the right tricyclic rings, allowing the molecules to interact with the G-quartet as well as the grooves and loops of G4.

### 4.4. Quindoline Derivatives

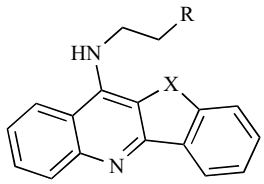
Several tetracyclic planar ligands have been synthesized, which have extended aromatic rings to fit in the G-quartet dimension. This class of compounds has a five-membered ring in the middle, fused in a linear arrangement to produce crescent-shaped molecule. The two types of quindoline derivatives, disubstituted (**19**-**21**) [49,50], and 11-subsitituted (**22**-**31**) [51,52] (Fig. **4**) are representatives of this class. All of these compounds have shown strong G4 stabilization and telomerase inhibition capacity but only modest G4 selectivity (2~3-fold) over duplex DNA. Biological studies showed that the 11-subsitituted quindolines could strongly suppress the functions of telomerase and *c-Myc* oncogene. The selectivity of compounds should be improved through structural modifications such as by selecting appropriate number and location of amino side chains.

### 4.5. Pentacyclic Acridinium Analogues

Further to the development of tetracyclic planar ligands, researchers have investigated the pentacyclic system (Fig. **5**), such as acridinium salts (**32**, **33**) [53], meridine (**36**) and asciddemin (**37**) [54]. Even without the side chains, most of these compounds have exhibited notable telomerase inhibition activity, especially some of the acridinium salts which have significant activity at submicromolar levels ($IC_{50}$ < 1 μM). A useful molecular feature for the notable activity of acridinium salts may be their partially positive charge at position 13-N of the acridine ring which acts as a ''pseudo'' potassium ion positioned above the centre of G-quartet in the region of high negative charge density [24]. Expanding the

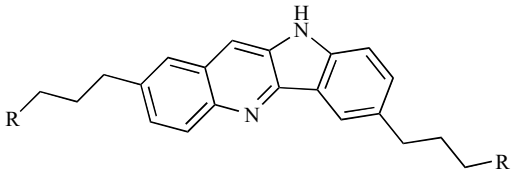


Disubstituted quindoline



11-subsitituted quindoline

**19**



Disubstituted quindoline

**20** R = 

**21** R = 

| NO. | X | R | NO. | X | R |
|---|---|---|---|---|---|
| **22** | NH | morphoryl | **27** | O | morphoryl |
| **23** | NH | $N(CH_3)_2$ | **28** | O | $N(CH_3)_2$ |
| **24** | NH | $CH_2N(CH_3)_2$ | **29** | O | $CH_2N(CH_3)_2$ |
| **25** | NH | OH | **30** | O | OH |
| **26** | NH | $CH_2OH$ | **31** | O | $CH_2OH$ |

**Fig. (4).** Structures of disubstituted quindoline and 11-subsitituted quindoline.

area properly and increasing the positive charge density of aromatic ring did increase the telomerase inhibition of these pentacyclic systems, however, these are not sufficient to gain significant selectivity as the aromatic ring simply stacks on the G-quartet and its size is still too small for the dimension of G-quartet. The results from competition dialysis assay, mass spectrometry and FRET analysis showed that all these compounds had modest G4 selectivity over other DNA sequences. Recently, based on the results from trisubstituted acridines, side chains have been introduced to the quinoacridinium pharmacophore in significant positions [55]. Some of these new acridinium salts, such as compounds **34** and **35**, have shown much lower cytotoxicity than the original compounds, due perhaps to decreased duplex DNA interaction. These studies suggest that appropriate side chains are also crucial for the G-quadruplex selectivity of pentacyclic system.

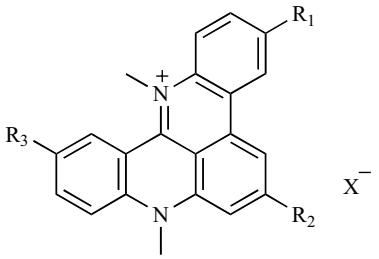
### 4.6. Dibenzophenanthroline Derivatives

Besides the pentacyclic acridinium derivatives, a series of pentacyclic dibenzophenanthroline ligands with crescent shaped arrangements and extended amino side-chains have been studied systematically (Fig. **6**) [56,57]. The N-methylation of quinacridine and its crescent ring shape in these compounds are considered crucial for their G4 binding affinity. The global charge created by the side chains has a strong influence on the binding, since highly cationic species can stabilize the quadruplex structure (**40** > **39** > **38**). Moreover, enhancement in affinity by the introduction of a third side chain may be gained from a synergistic effect between an optimized quadruplex interaction and an increase in the global charge (**41** > **38**), though the charge increase appeared to reduce the quadruplex selectivity as found with competition dialysis assay or FRET melting method. Compounds **38**, **42**, **43** and **44** was able to discriminate quadruplex against duplex DNA, while the disubstituted compounds with highly cationic side chains (**39** and **40**) or the trisubstituted compounds (**41**) had no such selectivity. Unlike the improved selectivity with the trisubstituted from the disubstituted acridines, here the introduction of a third side chain did not enhance the selectivity of quinacridine. The difference may arise from the location or length of terminal amine of the third side chain being incorporated into different pharmacophores. The side chains have a strong influence on fixation of the ligand in G-quadruplex and duplex DNA.

### 4.7. Fluoroquinolone Derivatives

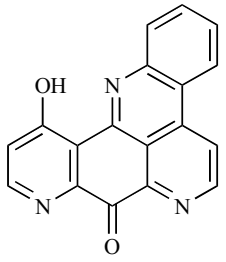
Fluoroquinolones (**45**) (Fig. **7**) are proven telomerase inhibitors, due probably to their interaction with G4 structures [58]. A series of fluoroquinolone derivatives have been synthesized with an extended aromatic conjugation system. Quinobenzoxazine A-62176 (**46**) is both a topoisomerase II poison and a catalytic inhibitor, while the extended hexacyclic fluoroquinophenoxazine QQ58 (**47**) is a strong G4 binder with no topoisomerase II poisoning activity, suggestive of its selectivity over duplex DNA [59]. However, the increase in aromaticity of the hexacyclic ligands resulted in poor selectivity for the G-quadruplex but higher topoisomerase II poisoning effects of fluoroquinoanthroxazines (**48** and **49**) [60].

A library of fluoroquinolone analogues with various aromatic conjugation systems and side chains has been designed and synthesized by Cylene Pharmaceuticals [61,62].



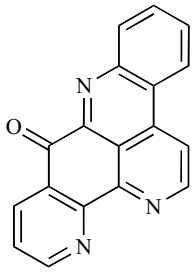
acridinium salts

| NO. | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| **32** | Me | Me | Me | $MeOSO_3$ |
| **33** | F | Me | F | $MeOSO_3$ |
| **34** | CH=CHCON$(CH_2CH_2)_2$O | H | H | I |
| **35** | CH=CHCON$(CH_2CH_2)_2$O | $(CH_2)_3OAc$ | Me | I |



meridine

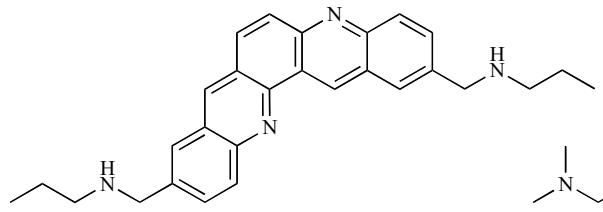
**36**



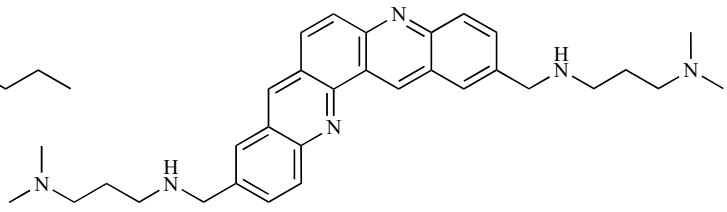
Ascididemin

**37**

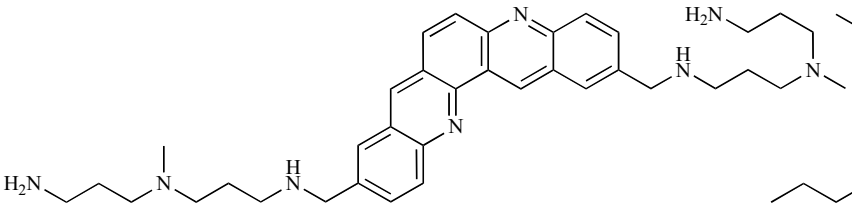
**Fig. (5).** Structures of pentacyclic acridinium analogs.



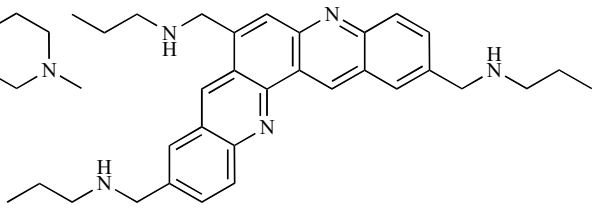
**38**



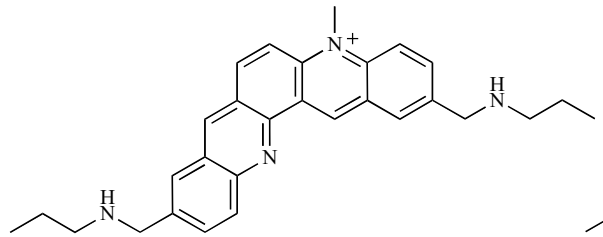
**39**



**40**



**41**



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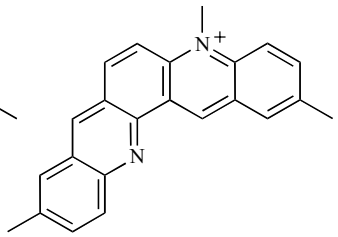

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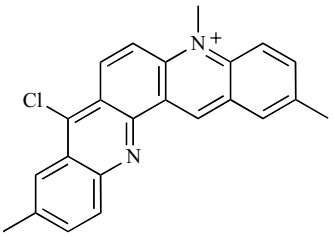
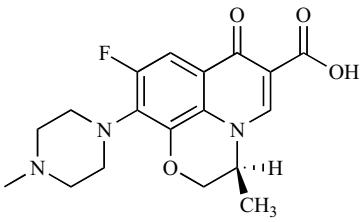


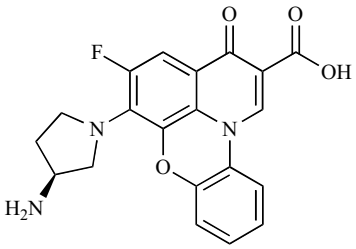
**44**

**Fig. (6).** Structures of dibenzophenanthroline derivatives.



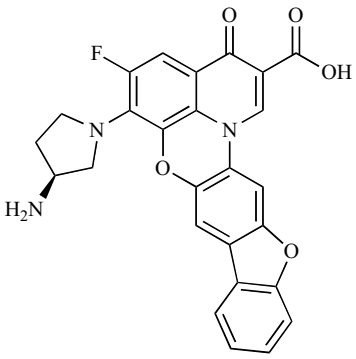
Levofloxacin

**45**



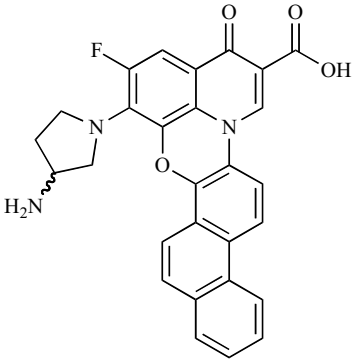
A-62176

**46**



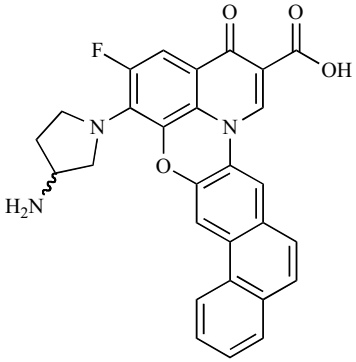
QQ58

**47**



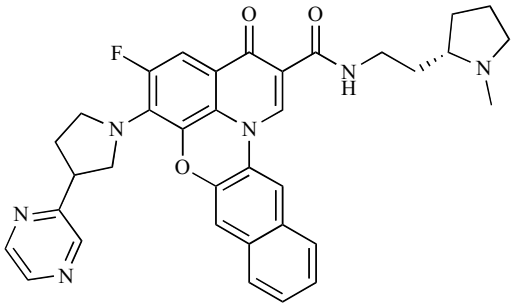
FQA-C

**48**



FQT-C

**49**



Quarfloxin (CX-3543)

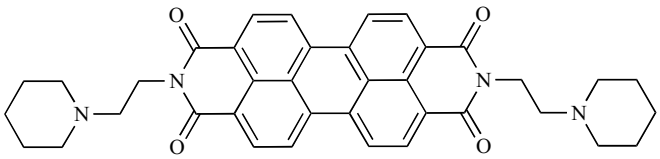
**50**

**Fig. (7).** Structures of fluoroquinolone derivatives.

The most promising in this library is quarfloxin (CX-3543) with pentacyclic system and two specific amino side chains (**50**) which is highly selective for specific G4 motifs (e.g., c-*Myc*). These two side chains are chiral substitutes and their asymmetric feature may allow for the quarfloxin to recognize the groove and loop regions of specific G4 motifs and gain the selectivity over other G4-structures or duplex DNA. However, quarfloxin did not show a direct inhibitory effect on c-*Myc* expression, suggestive of an alternative mechanism. It has been shown that quarfloxin disrupts the interaction between the nucleolin protein and a G4 DNA structure in the ribosomal DNA (rDNA) template, a critical interaction for rRNA biogenesis in cancer cells. In addition, it selectively induces the apoptotic cell death in cancers. It is not yet clear whether the mechanism of CX-3543 reflects its preference for rDNA quadruplexes over other quadruplex forms or just the high density of quadruplexes present in rDNA. Now, quarfloxin has entered Phase II clinical trial for cancer treatment.

### 4.8. Perylene Derivatives

Perylene derivatives, such as N,N'-bis[2-(1-piperidino) ethyl]-3,4,9,10-perylenetetra- carboxylic diimide (PIPER, **51**) (Fig. **8**), are classical agents used for selective interaction with G-quadruplexes. Previous studies [35,63] suggested a correlation between ligand aggregation and G4 DNA selectivity, and the derivatives that formed aggregates in a buffer had much higher G4 binding selectivity than the un-aggre-



PIPER

**51**

**Fig. (8).** Structure of PIPER.

gative species. The formation of aggregates as well as the binding selectivity was dependent on the pKa of their basic side chains. This was simply explained by that the aggregated ligand molecules became too large to interact with double-stranded DNA by inserting the ligand chromophore between the base pairs.

## 5. SELECTIVE G4 LIGANDS: MARCOCYCLIC SYSTEMS

### 5.1. Porphyrin Derivatives and Analogues

Porphyrins are well-known binding agents for duplex DNA. The planar arrangement of the aromatic rings in porphyrin analogues provides the potential for binding to G-quadruplexes by stacking on the G-quartets (Fig. **9**). The pioneer compound 5,10,-15,20-tetra-(N-methyl-4-pyridyl) porphine (TMPyP4, **52**) can stabilize different G-quadruplexes [64-66], and also bind non-specifically to all structural forms of DNA including single-stranded, duplex, triplex and quadruplex according to competitive equilibrium dialysis [67]. Another closely related porphyrin dye N-methyl mesoporphyrin IX (NMM, **53**), which is assumed to be anionic rather than cationic in an aqueous solution at physiological pH, has shown specific binding affinity to G4 structures with no apparent affinity to any other form of DNA [67,68]. These promising results from the early studies have motivated further research efforts for synthesizing new porphyrin derivatives with a variety of substituted groups to improve the binding affinity and selectivity, such as 5,10,15,20-tetra[4-hydroxy-3-(tri-methylammonium)methyl-phenyl]porphyrin (TQMP, **54**) [69] and pentacationic manganese(III) porphyrin (**55**) [70].

TQMP is a nonpyridinium cationic porphyrin with a phenol quaternary ammonium and has been shown by SPR analysis to bind 30-fold more strongly to quadruplex than to duplex DNA. TQMP may be more flexible than the rigid pyridinium porphyrin TMPyP4 owning to a higher steric selectivity. The introduction of a hydroxyl group may also be an important factor for increasing the interaction with G-quadruplexes by hydrogen bonding. These properties may favor its binding to G-quadruplexes in the grooves and increase its selectivity. Compared with TQMP, pentacationic manganese(III) porphyrin is even more promising with 10,000-fold selectivity for G-quadruplex over duplex DNA. This porphyrin contains a central aromatic core and four flexible cationic arms. The bulky cationic substituents surrounding the aromatic core, which prevent a close interaction with the double-stranded DNA structures, may be responsible for its low affinity for duplex DNA. Its high affinity for the G-quadruplex may be attributed to the interactions between the G-quartet and the porphyrin core, and between the grooves and/or loops and the flexible cationic arms.

Moreover, several modifications have also been made to the porphine core to generate a group of porphyrin analogues, such as tetramethylpyridiniumporphyrazines (TMPyPz) and their zinc complex (3,4-TMPyPz zinc(II), **56**) [71], and octa-cationic quaternary ammonium zinc phthalocyanine (ZnPc, **57**) [72]. Their cores are of higher aromaticity than the porphyrins. In addition, methylation of the pyridyl groups of tetrapyridinoporphyrazines or introduction of highly cationic side chains improved their quadruplex selectivity over duplex (>30-fold by **56** and >6-fold by **57**) due to electrostatic interactions with grooves or loops.

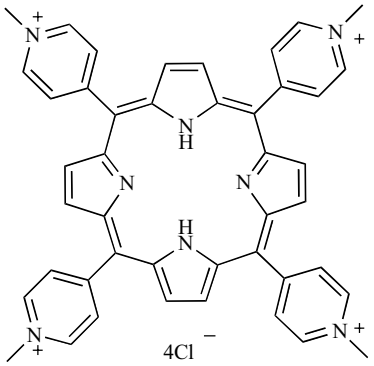
### 5.2. Telomestatin Analogues

Telomestatin (**58**) (Fig. **10**) is the first natural telomerase inhibitor of high potency ($IC_{50}$ = 5 nmol/L) owning to its ability to facilitate the formation of G4 structures or to stabilize the G4 structures [73]. It consists of one thiazoline ring and seven oxazole rings. Telomestatin appears to interact preferably with basket-type intramolecular G-quadruplexes than intermolecular quadruplexes, and induce the formation of the basket-type G-quadruplex from a random coil human telomeric oligonucleotide, even in the absence of monovalent cations such as $K^+$ or $Na^+$ [66,74,75]. Moreover, the specificity of telomestatin binding to intramolecular G4 structures is 70-fold over duplex according to polymerase stop assay [66]. The results from electrospray mass spectrometry and competitive FRET assay also indicate insignificant binding or stabilization of single-strand or duplex DNA by telomestatin [76,77]. However, this macrocyclic compound tends to occupy the whole G-quartet with little selectivity over different G4 structures.

Furthermore, a hybrid compound of telomestatin and TMPyP4, the selenium-substituted expanded porphyrin 5,10, 15,20-[tetra(N-methyl-3-pyridyl)]-26,28-diselenasapphyrin chloride (Se2SAP, **59**) has been synthesized [33]. Atom-by-atom superimposition and the electrostatic field-fit alignment studies show significant similarity between the oxazole ring of telomestatin and the bipyrrole ring of Se2SAP. As a consequence, Se2SAP should overlay very well with the entire G-quartet like telomestatin, and its charged N-methyl-4-pyridyl groups may also recognize the grooves/loops of different G-quadruplexes. SPR experiments have confirmed the selectivity of Se2SAP for the *c-Myc* G-quadruplex (~50-fold) over the duplex DNA. Se2SAP also showed a fairly high selectivity of binding to G-quadruplexes with a single lateral loop and the *syn-anti-anti-anti* arrangement of guanines in the G-quartets.

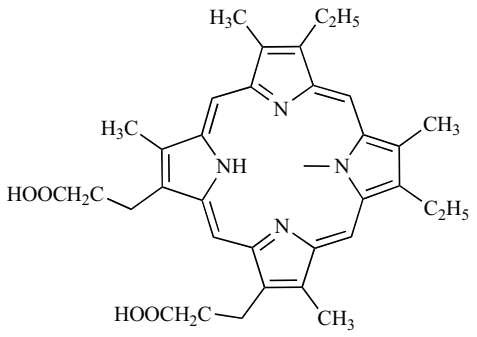
The unique selectivity observed in this macrocyclic system makes it a favorable molecular model for the design of G4 ligands, as for the synthesis of a series of oxazole-based peptides [78], cyclo[n]pyrroles [79], macrocyclic hexaoxazoles [80,81] and oligoamides [82]. Most of these compounds have shown significant binding affinity and high selectivity for G-quadruplex over duplex DNA. These compounds should have the similar interaction to that of telomestatin with G-quadruplexes by overlaying the entire G-quartet. Accordingly, the transformation of oligoamides from macrocylic (**60**) to helical structure (**61**) resulted in loss of selectivity.

Among the most popular macrocyclic ligands are oxazole-based peptide relatives (**62**-**65**) which contain three stereo amine side chains in the macrocyclic system [78]. These side chains are protonated at physiological pH and may be involved in stabilizing interactions with the grooves and loops of the quadruplex as well as the negatively charged phosphate backbone. SPR data indicate that inversion of all three stereocenters did not affect the recognition of homo-



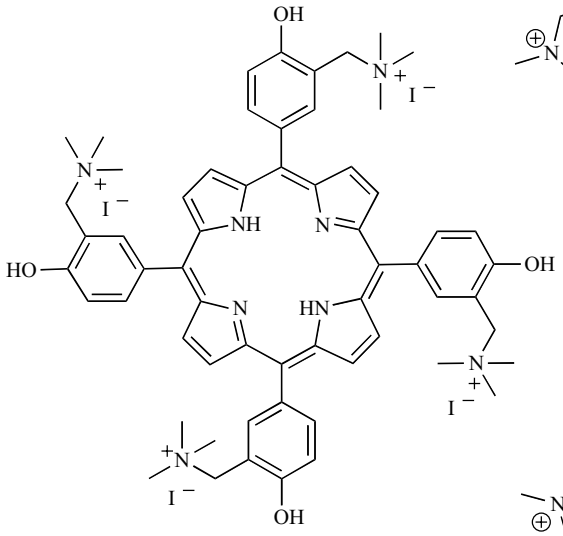
TMPyP4

**52**



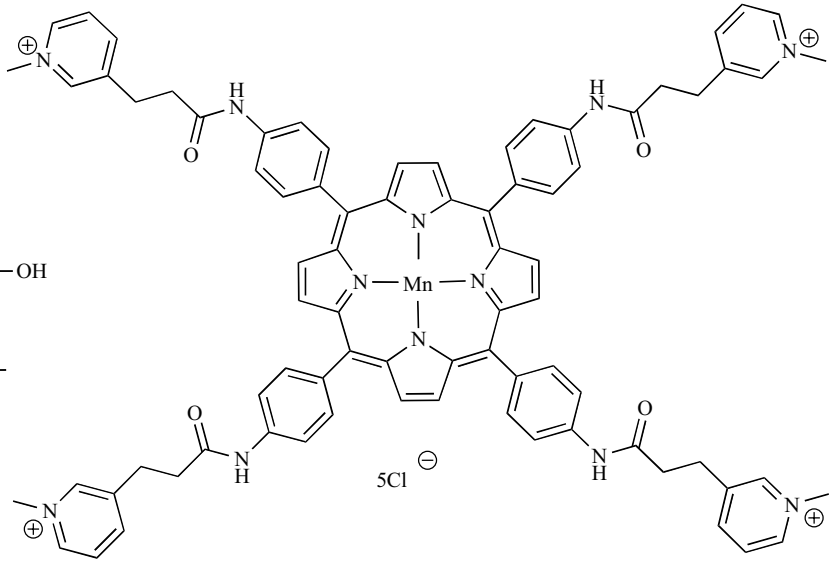
NMM

**53**



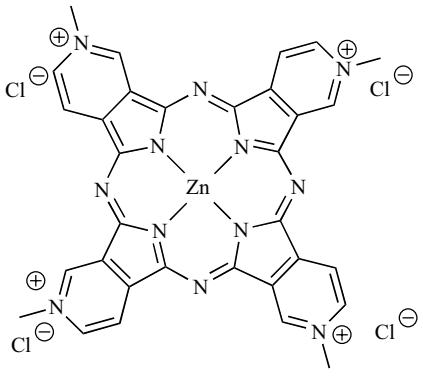
TQMP

**54**



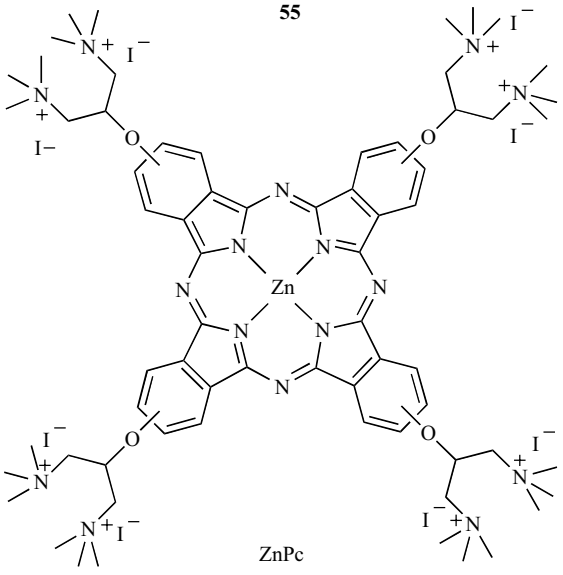
Pentacationic manganese(III) porphyrin

**55**



3,4-TMPyPz zinc(II)

**56**



ZnPc

**57**

**Fig. (9).** Structures of porphyrin derivatives and related analogs.

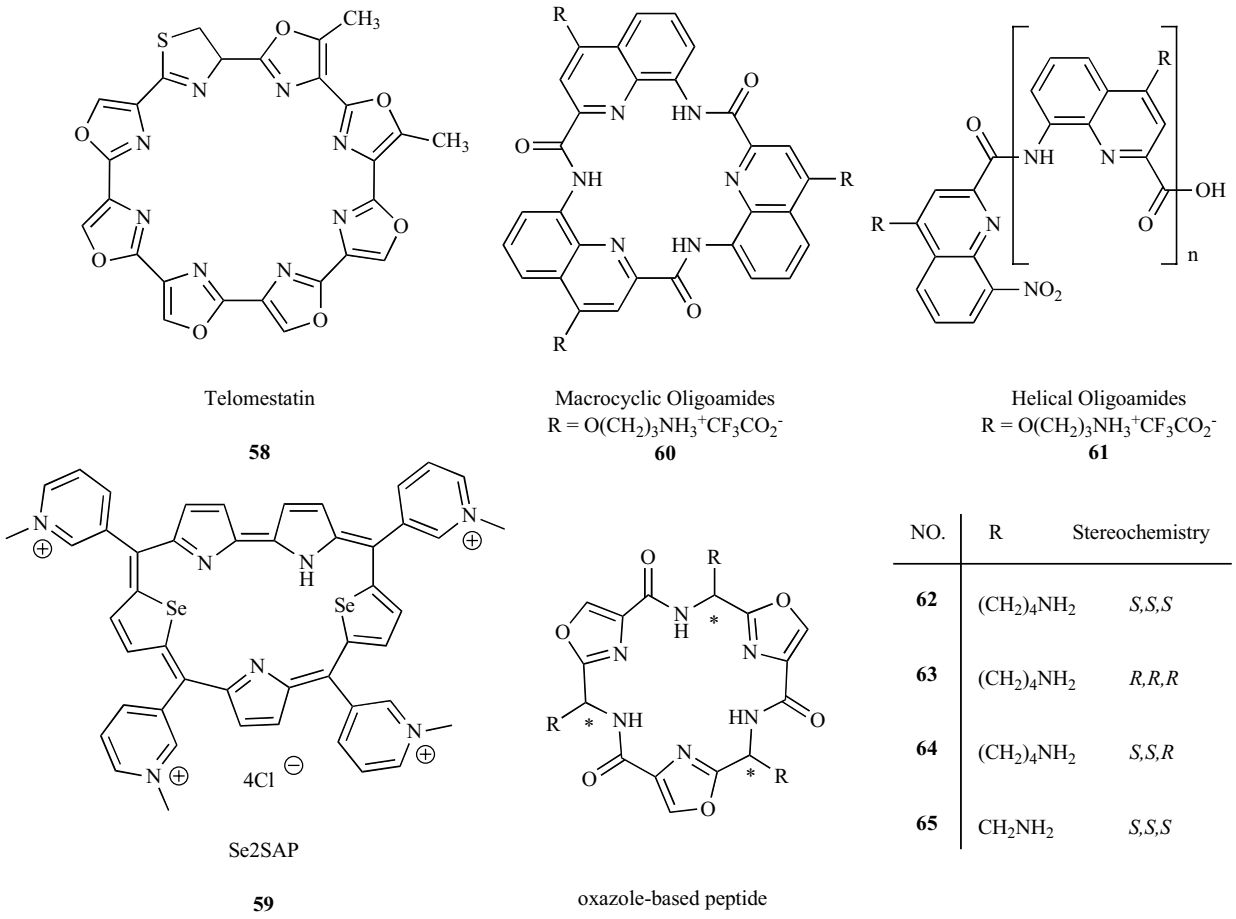



**Fig. (10).** Structures of telomestatin and related analogs.

chiral quadruplex structure (**62** and **63**), but inversion of only one stereocenter side chain on both faces of the macrocycle led to a 4- to 6-fold drop in binding affinity for diastereoisomer of *c-kit* and human telomeric quadruplexes (**64**). Moreover, for compounds **62** and **63**, it is noteworthy that introduction of side chains improved the *c-kit* selectivity 3-fold over human telomeric quadruplex. With shorter methylamine side chains (**65**), macrocycle showed a lower binding affinity for human telomeric quadruplex (4-fold), but a similar equilibrium binding to *c-kit* (7-fold).

The highly selective binding of macrocyclic ligands to G-quadruplex over duplex DNA is mostly attributed to their larger ring system, which overlap completely with the four guanines in the G-quartet, allowing the ligand molecule to occupy the whole area of the quartet region. However, targeting the G-quartet in the ligand design is insufficient to gain the selectivity between different quadruplex species, since this binding site is common to all quadruplexes. Findings from the study on oxazole-based peptide relatives (**62**-**65**) suggest that appropriate side chains are also crucial for designing macrocyclic ligands with selectivity for different G4 structures [78].

### 5.3. Cyclo Bis-Intercalant Analogues

Cyclo bis-intercalants belong to another macrocyclic system consisting of two intercalative-typed moieties bridged by polyamine linkers (Fig. **11**). For example, bisA (**66**) and BOQ1 (**67**) are made respectively of acridine and quinacridine cores linked with two diethylenetriamine arms [83,84]. These dimeric complexes have both improved affinity and selectivity for G-quadruplex compared to their respective monomers which have no selectivity between G4 and duplex DNA. The selectivity may be attributed to their cyclic framework which is unfavorable for their insertion into a DNA double helix.

The macrocyclic system can also be formed with a single aromatic core capped by a polyamine linker, such as the neomycin-capped aromatic structure (**68**-**71**) which is generated by intramolecular bis-tethering of neomycin on an aromatic core [85]. Neomycin is an aminoglycoside possessing several ammonium centers and can form multiple salt bridges and H-bonding contacts with nucleic acids. In addition, its 1,3-hydroxylamine motif is a potential recognition motif for the complexes of phosphate groups and of the Hoogsteen face of guanine. As a result, the derivatization of aminoglycosides with an intercalator system may lead to high-affinity ligands through simultaneous targeting the G-quartet and loops or phosphate residues. FRET-melting stabilization measurements showed that the neomycin-capped aromatic structures had moderate to high affinity for human telomeric quadruplexes, in correlation with the size of the aromatic moiety. Moreover, a FRET competition assay showed the poor binding ability of all macrocycles for du-

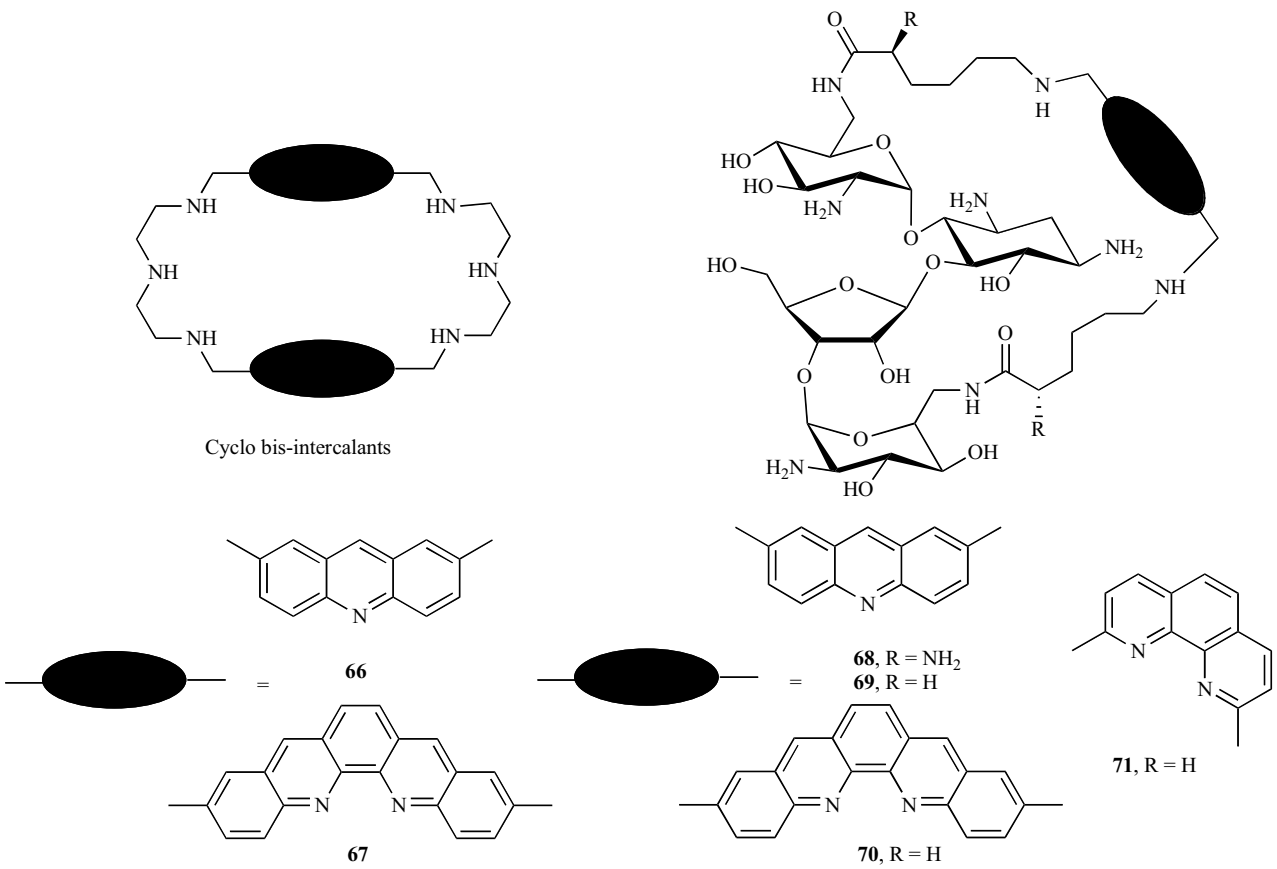



**Fig. (11).** Structures of cyclo bis-intercalants and related analogs.

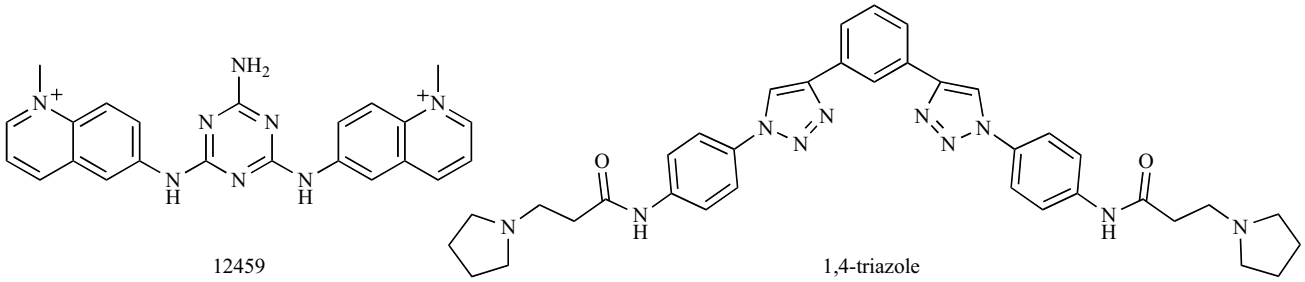
plex DNA and a clear binding preference for loop-containing intramolecular quadruplex structures over tetramolecular parallel G-quadruplex DNA, suggesting the structural significance of the loop for G4 recognition.

## 6. SELECTIVE G4 LIGANDS: TRIAZINE ANALOGUES

The investigation of interactions between G-quadruplex and non-planar compounds begun with studies on the carbocyanine dye 3,3'-diethyloxadicarbocyanine (DODC) and groove binder distamycin. Unlike its binding to the minor groove of duplex DNA, distamycin stacks on both ends of a G-quadruplex since the planar G-quartets provides an ideal platform for the stacking of aromatic rings in distamycin [23]. The higher aromaticity triazine derivatives may assume the same binding mode (Fig. **12**). In this series, 12459 (**72**) is the most selective G4 interactive compound which showed a 25-fold telomerase inhibition over the Taq polymerase inhibition according to the TRAP$^3$-G4 assay [86]. Other structures are analogues possessing the similar butterfly-shape, such as the bisquinolinium compounds with two quinolinium moieties connected through a 2,6-pyridodicarboxamide unit (**73** and **74**) which displayed modest affinity and selectivity for G-quadruplex. Expanding the aromaticity and planarity from bipyridine to phenanthroline core (**75** and **76**) significantly enhanced their interaction with G-quadruplex but no obvious interaction with duplex DNA [87]. The stacking interaction with G-quartet has been confirmed as the chief binding mode of these compounds by competitive FRET melting assay. It is noteworthy that the wings of these butterfly-shape compounds can be constructed with diverse components such as amino side chains in 1,4-triazole (**77**) [77] and biarylpyrimidine derivatives (**78**) [88], all having high selectivity for G-quadruplexes. The high selectivity of these ligands is attributable to their adaptive structural feature arising from the rotatable bonds, which allows for the ligands to adopt different conformations so as to fit the shape of groove and loop regions of G-quadruplex while maintaining the rigidity. As a result, the design of non-coplanar molecules is a feasible approach for selective quadruplex ligands over duplex DNA.

## 7. SELECTIVE G4 LIGANDS: PEPTIDES AND PROTEINS

The design of proteins that can recognize DNA with high affinity and sequence specificity has been a major goal in the study of protein-nucleic acid interactions. It has been reported that a zinc finger protein isolated from the phage library binds to single-stranded human telomeric G4 structure with an affinity comparable to that of natural transcription factors, and with strong discrimination against the duplex DNA [34]. This finding has motivated the efforts to exploring various synthetic peptides and peptide-ligand hybrids for specific interaction with G-quadruplex (Fig. **13**). The SPR data showed that tetrapeptides (**79**-**81**) bind weakly to G-quadruplex but with notable preference for quadruplex over double-stranded DNA, e.g. up to 5-fold selectivity by the FRHR (**81**) [89]. Conjugation of these selected peptides with

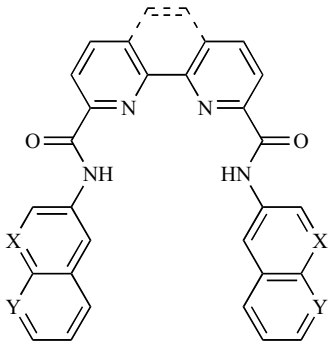


12459

**72**

1,4-triazole

**77**

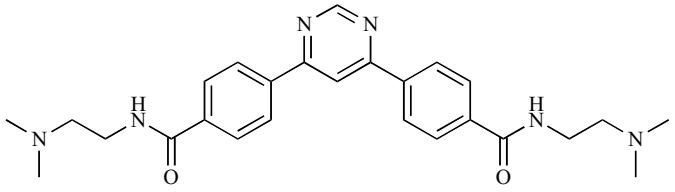


**73** 2,2'-bipyridine, X = $NCH_3^+$, Y = CH

**74** 2,2'-bipyridine, X = CH, Y = $NCH_3^+$

**75** 1,10-phenanthroline, X = $NCH_3^+$, Y = CH

**76** 1,10-phenanthroline, X = CH, Y = $NCH_3^+$



biarylpyrimidine

**78**

**Fig. (12).** Structures of triazine derivatives and related analogs.

a hemicyanine (**82**-**84**) or acridine core (**85**-**87** and **91**-**93**) improved their affinities and selectivity with G-quadruplex, especially those with the FRHR arm (**87** and **93**) [89,90]. Peptide shortening (**88**-**90** and **94**-**96**) resulted in a dramatic decrease in selectivity, suggesting that the FRHR tetrapeptide can interact with the loops in G4 structures but its steric bulky conformation cannot be accommodated by the duplex DNA. These results have also been confirmed by molecular modeling. However, peptide shortening in acridone conjugates (**88**-**90**) actually resulted in a slight enhancement in affinity with both duplex and G-quadruplex.

## 8. OTHER G4 BINDERS

Besides the metal porphyrins reviewed in an early part, numerous other metal complexes have been reported as selective G4 binders, such as platinum–quinacridine hybrids [91], perylene-metal complexes [92], square-planar nickel (II) complexes [93] and metal-terpyridine complexes [94] (Fig. **14**). Among them, the square-planar nickel (II) complex (**97**) with amine side chains may be the most selective compound. The selectivity may arise from the electrostatic interaction with the central ion channel or loops of G-quadruplex by the metal cations. In addition, the geometry of the metal center may strongly influence the ability of a compound to discriminate quadruplex against duplex-DNA. For example, the pyramidal shape of Cu cation in compound **98** can impede its intercalation within duplex DNA.

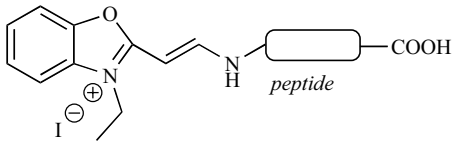
There are still other selective compounds, such as expanded ethidium [95], berberine derivatives [32], steroid FG, and a funtumine derivative substituted with a guanylhydrazone moiety [96]. Although these compounds may not fit in any of the structural categories reviewed above, they share the similar design principles for selective interaction with G-quadruplex.

## CONCLUSION

This review presented a large collection of selective G4 ligands. Their binding affinity and selectivity depend on properties of both the chromophores and the substituted groups, particularly the shape and electron density of the chromophore, the size, number and location of substituted groups. Besides the most preferred amine side chains, other substituted groups have also shown notable selectivity, including peptides, aminoglycosides, anilino groups, quinolinium moieties and metal complexes. According to instrumental measurements and computational simulation of G4 structures and G4-ligand complexes, the target binding sites for selective G4 ligands include the G-quartet surface, grooves and loops, and the possible modes of interaction include stacking (onto the planar ends), electrostatic attraction, hydrogen bonding and other molecular forces.

Substituted groups are crucial for the ligands that belong to fused aromatic system to achieve G4 selectivity over duplex DNA. An emerging trend is toward the enhancement of

| | | |
|---|---|---|
| **79** | $NH_2$-RKKV-COOH | $NH_2$-Arg-Lys-Lys-Val-COOH |
| **80** | $NH_2$-KRSR-COOH | $NH_2$-Lys-Arg-Ser-Arg-COOH |
| **81** | $NH_2$-FRHR-COOH | $NH_2$-Phe-Arg-His-Arg-COOH |

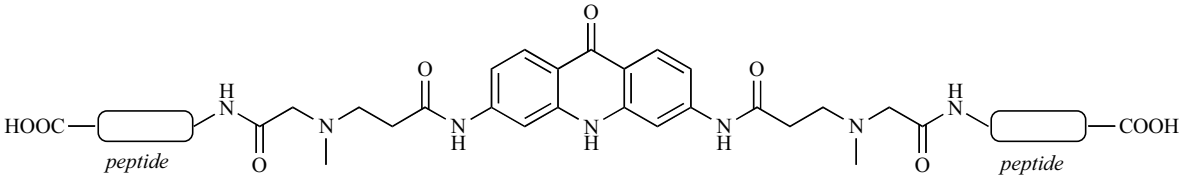



Peptide sequences

**82** NH-RKKV-COOH

**83** NH-KRSR-COOH

**84** NH-FRHR-COOH




Peptide sequences

**85** NH-RKKV-COOH

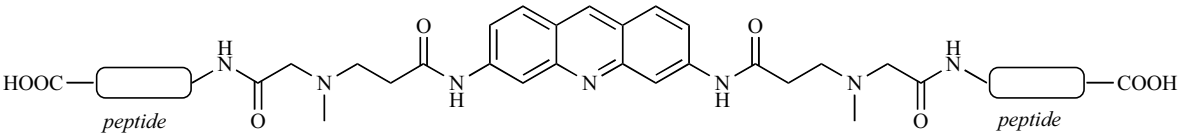
**86** NH-KRSR-COOH

**87** NH-FRHR-COOH

**88** NH-FRH-COOH

**89** NH-FR-COOH

**90** NH-F-COOH




Peptide sequences

**91** NH-RKKV-COOH

**92** NH-KRSR-COOH

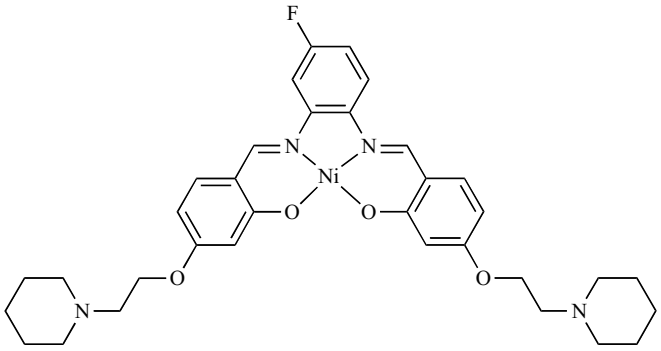
**93** NH-FRHR-COOH

**94** NH-FRH-COOH

**95** NH-FR-COOH

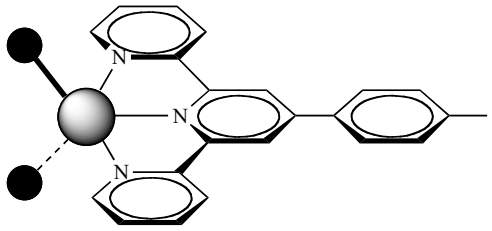
**96** NH-F-COOH

**Fig. (13).** Structures of peptides and peptide-ligand hybrids.




Square-planar nickel (II) complex

**97**




Metal = $Cu(NO_3)_2$

**98**

**Fig. (14).** Structures of metal complexes.

grooves and loops recognition by the introduction of additional substitutes on the coplanar chromophore. While the individual side chains may have different affinities for a quadruplex, their synergistic effect is also important for differential interaction with different G4 structures. An important characteristic of the substituted groups is their selective interaction with the structurally more complex loop or groove regions in addition to π-π stacking on the G-quartet. Most of the G4 ligands have extended planar chromophores facilitating their stacking onto the planar ends of a G-quadruplex. An elegant exemplification for this design principle is provided by the macrocyclic compounds. Further more, several non-coplanar molecules may be more promising candidates in the search for high selective G4 ligands with drug-like scaffolds. Clarification of their binding modes with G-quadruplex is warranted for better understanding of their selectivity.

Over the last few years of less than a decade, remarkable progress has been made in the development of selective G4 ligands and the understanding of their interaction with G4 and structure-activity relationship. Some of the G4 ligands have shown excellent affinity and selectivity for G-quadruplex and significant telomerase inhibition or suppression of the transcription activity of oncogenes, and a few have entered clinical trials for cancer therapy. These achievements have enlightened the promising prospects of G4 ligands as anticancer agents with reduced side effect and toxicity. With increased knowledge of ligand-quadruplex interactions, we can anticipate that more effective G4 ligands can be developed for cancer therapy in the near future.

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## ABBREVIATIONS

| | | |
|---|---|---|
| ESI-MS | = | Electrospray Ionization Mass Spectrometry |
| FRET | = | Fluorescence Resonance Energy Transfer |
| G4 | = | G-quadruplex |
| SPR | = | Surface Plasmon Resonance |
| $T_m$ | = | Melting Temperature |
| TMPyP4 | = | 5,10,15,20-tetra-(*N*-methyl-4-pyridyl)porphine |

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