The Design of G-quadruplex Ligands as Telomerase Inhibitors

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Abstract: Guanine-rich repetitive DNA sequences are of particular importance at the ends of chromosomes, where they are associated with a number of proteins to form telomeres. Their function is in large part to protect chromosomal ends from unwanted degradation and chromosomal fusions, although in normal somatic cells telomeres progressively shorten, eventually becoming non-proliferating and consequently these cells have a finite lifetime. By contrast tumour cell telomeres are maintained in length so that tumour cells are effectively immortalised. The reverse transcriptase enzyme telomerase is activated in over 80% of tumour cells, and it undertakes the synthesis of further telomeric DNA repeats, so directly maintaining telomeres. The inhibition of telomerase leads to the senescence and eventual apoptosis of tumour cells, and thus telomerase is an attractive target for selective chemotherapy. This review describes an approach to the inhibition of telomerase that involves the folding of telomeric DNA into a four-stranded quadruplex structure, held together by Hoogsteen hydrogen-bonded arrays of guanine bases. The formation of a quadruplex structure at the 3' end of telomeric DNA effectively hinders telomerase from adding further repeats. A number of small-molecule ligands are described that stabilise quadruplex formation, and which result in telomerase inhibition. Implications for anti-tumour therapy with such molecules are discussed, and the particular challenges and problems discussed.

INTRODUCTION

Guanine-rich sequences of DNA are found primarily at the ends of chromosomes, as telomeric protein complexes [1]. They are also present in a number of human genes [2], notably in promoter sequences of several proto-oncogenes (such as c-*myc*), in immunoglobin switch regions, and are implicated in the hereditary disease fragile X syndrome. Such sequences share the common ability of being able to form higher-order DNA structures [2] in contrast to the standard B-form duplex DNA of the overwhelming majority of genomic DNA. Few of these guanine-containing structures have been unequivocally characterised at the three-dimensional level, not least on account of their diversity and complexity. This is true even of the best-studied G-rich sequences, of telomeric DNA, which consist of tandem repeats of short sequences such as d(TTAGGG), the repeat in mammalian cells.

Telomeres are currently of considerable interest in view of the demonstration [3] that tumour cells are fundamentally distinct from normal human somatic cells in their ability to maintain telomere length at a constant level (ca 3-6kb, depending on tumour cell type). By contrast telomeres in normal cells shorten with each round of cell division as a consequence of the inability of DNA polymerase to fully replicate the ends. Consequently normal cells have a finite lifespan whereas tumour cells are effectively immortalised. Telomere maintenance in 80-85% of tumour cells is achieved by the action of the reverse transcriptase enzyme telomerase, which catalyses the synthesis of further telomeric DNA repeats. The critical catalytic subunit of telomerase is not expressed in normal cells [4], although the RNA domain is widely expressed in many cell types. This feature of telomerase thus suggests that it can be an exceptionally attractive target for cancer therapy, at least in principle [5, 6].

Telomeres in tumour cell are on average significantly shorter than those of somatic cells. Thus, inhibition of telomerase can result in telomere attrition to a critically short telomere length (leading to the characteristic senescent state when cell growth irreversibly ceases, which is then often the precursor to apoptosis). The situation may well be much more favourable than this. It has recently been suggested that within a population of cells there are large variations in telomere lengths, and moreover that the shortest telomeres are actually the most sensitive and critical for cell viability [7]. This suggests in turn that disruption of the delicate balance of telomere maintenance in tumour cells can be readily achieved. It has been shown with both dominant-negative telomerase transfection studies [8] and antisense experiments [9], that telomerase inhibition leads to telomere shortening and ultimately to selective senescence and apoptosis of tumour cells, a key demonstration of proof of principle.

G-QUADRUPLEXES AS TARGETS FOR ANTI-TELOMERASE THERAPY

Telomerase presents a diversity of possibilities for inhibition. Those being most actively explored are: (i) the catalytic site [10], (ii) its (RNA) template on which further telomeric DNA is synthesised [9], usually by means of an antisense approach (iii) mutation of the RNA domain, and

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thus disruption of its interactions with the catalytic domain [11], and (iv) the telomeric DNA substrate itself [12]. This last category is the subject of the present review.

The telomeric DNA primer is required to be single-stranded in order to participate in the initial step of telomerase-catalysed 3'-end extension [13]. This involves the recognition of the three terminal bases by the RNA template (within the RNA domain), by means of standard Watson-Crick hydrogen bonding. The 3'-terminal 150-200 bases of telomeric DNA are single-stranded, so template hybridisation normally occurs without hindrance. Folding and stabilising the DNA substrate into a non-single-stranded conformation by means of a stabilising ligand results in effective telomerase inhibition, with telomere extension ceasing beyond the first 3-4 telomeric repeats [14], suggesting that these form a minimal arrangement, beyond which further extension cannot occur.

Association of telomeric DNA sequences is driven by the formation of guanine-quartets, and this can occur either on an inter- or intramolecular basis [15, 16]. The resulting G-quadruplex (G4) structures exhibit a diversity of features. Common elements are:

- Four discrete DNA strands, formed from one or more DNA sequences.
- The presence of several layers of π-π stacked G-quartets, linked by phosphodiester backbone.
- The presence of non-G nucleotides, notably T and A, which can form non-helical loop regions.
- The requirement for metal cations to impart stability, with K^+ followed by Na^+ being the most effective.

The diversity shown by those structures that have been determined to date ranges from the simple parallel strands in the intermolecular complex formed by four strands of d(TGGGGT) [17], to the anti-parallel dimer formed by two strands of d(GGGGTTTTGGGG) [18]. The intramolecular quadruplex formed by the 22-mer sequence $d(AGGG(TTAGGG)_3)$, ie from almost four human telomeric repeats, has been reported [19] to form a structure as a Na^+ complex with anti-parallel strands and three d(TTA) loops stacked onto the ends of the G-quartets. This structure has been used by several groups as a starting-point for structure-based design studies in the search for G-quadruplex ligands as effective telomerase inhibitors (see below). However a recent crystallographic study in this laboratory has shown that this sequence in the presence of K^+ adopts a radically different structure with all the strands parallel and the resulting diagonal d(TTA) loops oriented away from the G-quartets [20]. This has clear implications for the design of molecules that interact with both G-quartets and the loops.

The targeting of G4 structures does not require that they are necessarily present at the 3' end of telomeres in the absence of ligands. There is evidence that ligands such as PIPER (see below) can accelerate quadruplex formation and folding, acting in a chaperone-like manner [21]. This suggests that there is a dynamic equilibrium at telomere ends between single-stranded and various types of folded telomeric repeats, including the looped structures visualised in electron microscopy studies [22]. Telomerase itself will force this equilibrium towards single-strandedness at replication, since only then can template hybridisation and the synthesis of telomeric repeats on to 3' ends take place.

Until recently the existence of G4 telomeric structures *in vivo* was controversial. Several studies have identified G4-specific proteins in both human [23] and yeast [24] cells. Although their roles remain to be clarified, it is likely that they are involved in recombination and the resolution of DNA topological problems. It may be significant that telomere length can be maintained in the absence of telomerase by ALT (alternative lengthening of telomeres) mechanisms, which occur in 10-15% of tumours, such as osteosarcomas), probably by recombination processes [25]. These may involve intermediates such as G4-type structures, with specific helicases probably responsible for their unwinding [26]. It has recently been shown that the unwinding of two such human helicases, from Bloom's and Werner's syndrome, is inhibited by G4 ligands [27], as is the analogous helicase from yeast [28]. Thus the stabilisation of quadruplexes may well be of more general significance in the disruption of telomere maintenance [29] than solely in the mediation of telomerase inhibition, and we suggest that G4-interactive ligands are likely to be active in ALT tumours as well as telomerase-positive ones.

Telomerase activity can be estimated by the extent to which a telomeric DNA primer is elongated. It is possible to employ partially-purified enzyme from cellular extracts in a direct assay [14], although this requires the use of high specific-activity ^{32}P-labelled primer. A widely-used alternative is the TRAP (Telomere Repeat Amplification Protocol) assay, which incorporates PCR to amplify the extension products. This is readily adapted to quantitation, and in particular to obtain EC_{50} values (the effective concentration for 50% inhibition) for ligands from dose-response studies. It is necessary to undertake control experiments to check that a given ligand does not itself inhibit the Taq polymerase enzyme used in PCR. It is not possible to quantitatively compare reported activities for the various ligands discovered to date, not least in view of variations in TRAP assay protocols.

The goal for G-quadruplex ligands must be to optimise selectivity and potency to this target, and to minimise or even eliminate interactions with other DNAs, especially duplex DNA. Such interactions are major contributors to cytotoxicity (as measured by IC_{50} values in conventional short-term growth inhibition assays for cytotoxic agents), which are probably mediated via DNA topoisomerase enzymes. Thus a quadruplex inhibitor for which $EC_{50} \sim IC_{50}$ will in practice kill cells by cytotoxic mechanisms before effective telomerase inhibition can occur. Selective telomerase inhibition requires the ratio EC_{50}/IC_{50} to be at least 10, and preferably considerably greater than this. Unfortunately not all studies have reported growth inhibition data on telomerase inhibitors, so that in these instances their potential for cellular studies cannot be assessed. A plausible working assumption that the strength of G-quadruplex ligand binding is directly correlated with extent of

telomerase inhibition has been supported by several studies (see below). This in turn has led to the increasing use of high-throughput quadruplex-binding assays to find lead compounds. Such assays can be real or *in silico*, the latter using molecular modelling and simulation methods to estimate interaction energies. Ligand binding to G-quadruplexes in solution can be evaluated by a range of methods; T_m measurements, uv/vis Scatchard plots, competition dialysis, fluorescence resonance energy transfer (FRET) measurements, gel retardation methods, and surface plasmon resonance (SPR) have all been used to good effect.

Telomerase inhibitors are likely to be required for longer-term administration than is usual for anti-cancer agents. Thus, from the outset of a ligand discovery programme, it is necessary not only to optimise the drug-like features of potential telomerase inhibitors (uptake, distribution and metabolism), using ADME-type approaches, but also to build in features for potential oral bio-availability and minimal toxicity. There is also a need to develop simple and versatile, high-yielding economically-viable synthetic routes in order to rapidly generate sufficient amounts of material for full *in vivo* studies and eventual clinical trials. Such studies are beyond the scope of this review, but the unique nature of the role played by telomerase in tumorigenicity will undoubtedly require specifically designed long-term animal studies as part of a strategy to develop clinical candidate molecules. Such molecules will necessarily have minimal toxicity compared to conventional DNA-binding agents. Clinical trials will also require the definition of reliable molecular markers. Changes in telomere length are at first sight the obvious choice, with short telomeres (which are especially sensitive to cell viability [7]) being a major focus.

COMPUTER MODELLING OF LIGAND-G4 COMPLEXES

The prerequisite for a rational drug design programme based on molecular modelling studies, is structural information on the target molecule. G-quadruplexes can be formed in several ways, although it is entirely reasonable to assume that an intramolecular structure is especially relevant to the folding of 3' end telomeric DNA. The initial finding of an amidoanthraquinone derivative as a telomerase inhibitor [14], also showed that approximately four d(TTAGGG) repeats are required to be formed before inhibition occurs. The NMR structural model [19] subsequently used in a number of studies has the four-repeat sequence d[AGGG$(TTAGGG)_3$], so is a relevant model to be used, that is consistent with this finding. Modelling studies [39, 41] suggested that a high-affinity ligand binding site can be formed at one end of the stack of G-quartets (Figure **1**), by opening up the diagonal T_2A loop at the 5'-AG step. This loop is stacked on top of the terminal G-quartet. In contrast the crystal structure of the same sequence [20], has all the loops diagonally arranged, so that they are situated in the grooves and are not stacked over the G-quartet ends. The latter are thus more open to ligand binding, and present a large open surface. There is no crystal structure of such a complex reported to date, but several molecular modelling and NMR studies all concur in demonstrating that planar aromatic chromophores stack on the exterior of the G-quartet stack (Figure **1**), and do not intercalate within it [30-32]. However a full NMR analysis of a complex involving the human intramolecular quadruplex has not been reported to date, as a result of line-broadening effects.

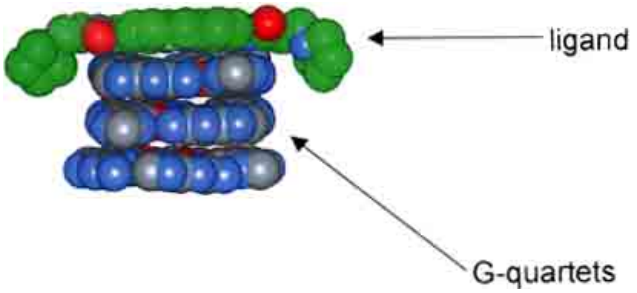

Fig. (1). Schematic of the interaction of a disubstituted ligand with a G-quadruplex. The planar chromophore part of the ligand is shown stacked onto the terminal G-quartet of the quadruplex.

Ligand interactions with quadruplexes can involve three principal categories of binding site, regardless of the structural details of an individual target:

- The extended planar G-quartet surface, with planar heteroaromatic chromophores stacking on the surface by means of π-π interactions.
- The grooves between adjacent phosphodiester chains, which can vary in dimensions.
- The non-helical loops, such as d(TTA) in human telomeres.

Almost all G-quadruplex ligands reported to date have extended planar chromophores, and stacking on the G-quartet end(s) is undoubtedly an important factor in their binding. We have used computer modelling methods [30, 44] to examine these interactions for a representative set of ligands, and the various low-energy stacking arrangements are shown below. In general the extent of stacking correlates qualitatively with quadruplex binding and telomerase inhibition.

TRICYCLIC G4 LIGANDS

Historically ethidium bromide (Figure **2**) was the first compound to be shown to interact with G-rich DNA sequences [33], although the nature of the interaction was not revealed and telomerase inhibition was not reported. A pioneering *in silico* search [34] was used to find the carbocyanine derivative DODC from a library of compounds (Figure **2**), which was also shown to bind to a G-quadruplex in solution. A subsequent competition dialysis analysis [35] has suggested that both DODC and ethidium actually bind to duplex and triplex DNA with greater affinity. This is in accord with a systematic study [36] of the interactions of

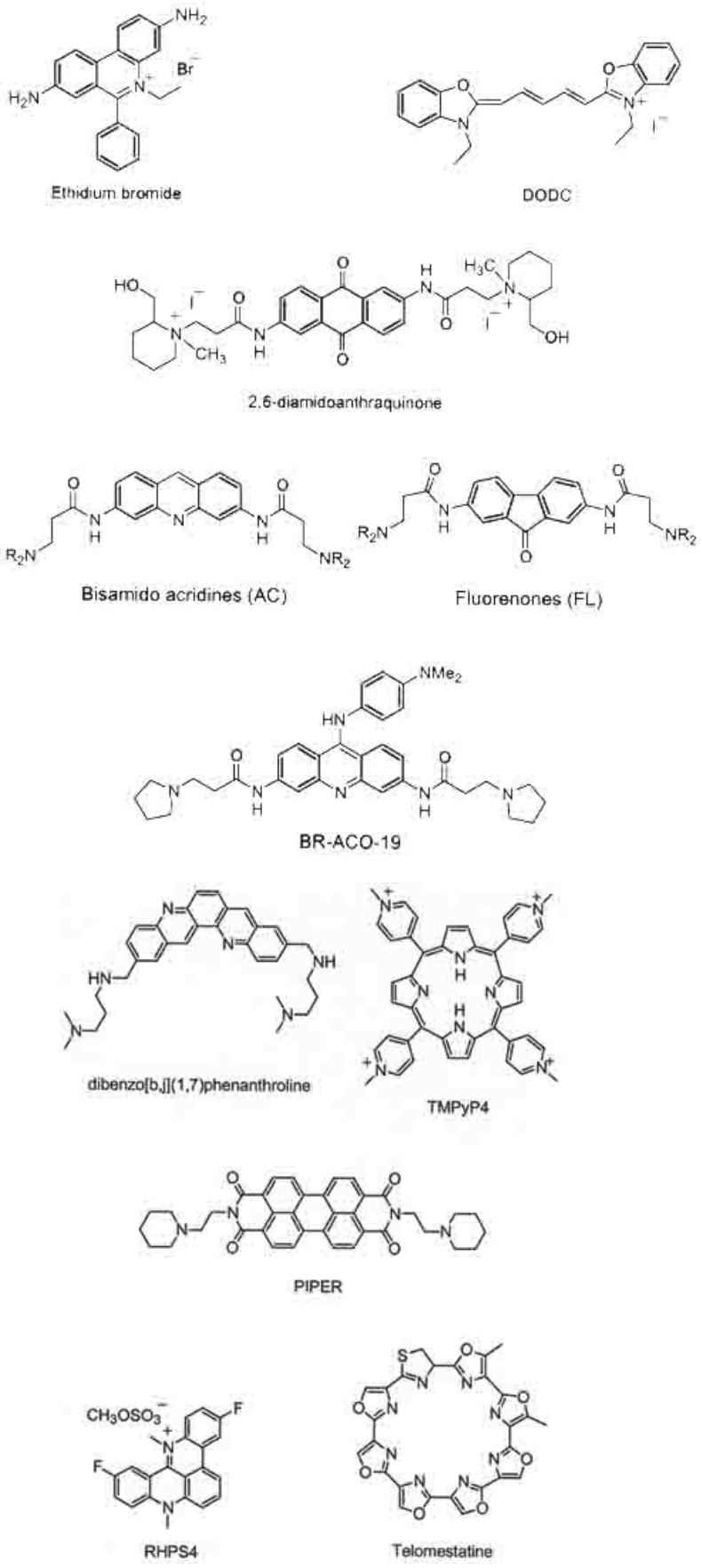

Fig. (2). Structures of G4-interacting ligands.

ethidium and several derivatives with both inter- and intramolecular quadruplexes. This has also revealed that strength of ethidium binding to a quadruplex depends on the nature of the quadruplex, with binding to the human intramolecular four-repeat quadruplex being weak. These differences can be presumed to be a consequence of differences in geometry between the various binding sites in different quadruplex folds.

The first small non-nucleoside molecule to be reported as a telomerase inhibitor was the 2,6-diamidoanthraquinone shown in Figure **2** [14]. The rationale for the amido functionalisation has been that these are involved in the overall delocalised area of the chromophore, thereby potentially enhancing π-π stacking interactions with DNA bases. This type of substituted anthraquinone had been first described as a cytotoxic compound [37], and was subsequently shown to have some preference for triplex DNA [38]. From that starting point, a large number of compounds, notably the 1,4, 1,5, 1,8, 2,6 and 2,7 regioisomers have been designed, synthesised and evaluated as telomerase inhibitors in order to find the best compromise between telomerase inhibition and inhibition of cell growth by cytotoxic mechanisms [39]. Their synthesis starts from the appropriate n,m-diaminoanthraquinone. The 1,4, 1,5 and 2,6 regioisomers are commercially available; the 1,8 one is not but can be obtained via nucleophilic aromatic substitution of the dichloro derivative with sodium phthalimide and later hydrolysis (Figure **3**). These intermediates can be transformed by a two-stage procedure into the final aminoalkylamido derivatives. The first step involves the reaction of an appropriate chloro-acid chloride with a diamino substituted aromatic ring. The second step is an aminolysis of the dichloro derivative with a secondary amine.

The nature of the terminal amino substituent, the alkyl side-chain linker length and the effect of regioisomerism on telomerase activity have all been systematically studied within these series. Two substituents are always required, each of which needs a terminal cationic group. Modelling shows that the anthraquinone chromophore effectively stacks onto two guanines, with the side-chains protruding into grooves (Figure **4a**). A number of compounds show telomerase EC_{50} inhibitory values in the low micromolar region regardless of the pattern of substitution. However, this level of activity is too close to their IC_{50} values in various tumour cell lines (Tables 1, 2) and amidoanthraquinones were therefore considered to be unsuitable for detailed cellular or *in vivo* studies.

Fluorenones have an overall structural similarity to anthraquinones, albeit without having quinoid character. It was suggested [40] that cytotoxicity arising from the redox properties of quinones could be avoided, and thus that fluorenones would be less cytotoxic. The telomerase activity and cytotoxicity of some fluorenones is given in Tables 1 and 2. This shows a loss of telomerase activity compared to amidoanthraquinones, even though cytotoxicity is diminished. Thus the ratio EC_{50}/IC_{50} remains ~1. The loss of telomerase activity was attributed to the change in the angular disposition of the side chains compared with the analogous 2,6-anthraquinone derivative. A good fit into the putative G4 binding site can only be achieved by distortion of the site geometry and therefore there is a consequential loss in net binding energy. The synthetic route to fluorenones is similar to that for the anthraquinone inhibitors. Reduction of 2,7-dinitrofluorenone followed by acylation with 3-chloropropanoyl chloride and substitution with a range of secondary amines provides the fluorenone derivatives in good yield (Figure **5**).

The notion of introducing a positive charge in the central ring of the chromophore that would complement the channel of negative electrostatic potential that runs through the centre of a quadruplex, has led to the study of a series of 3,6-bisamidoacridines [41-43] and evaluation as telomerase inhibitors. Table 2 shows that they do not show significant

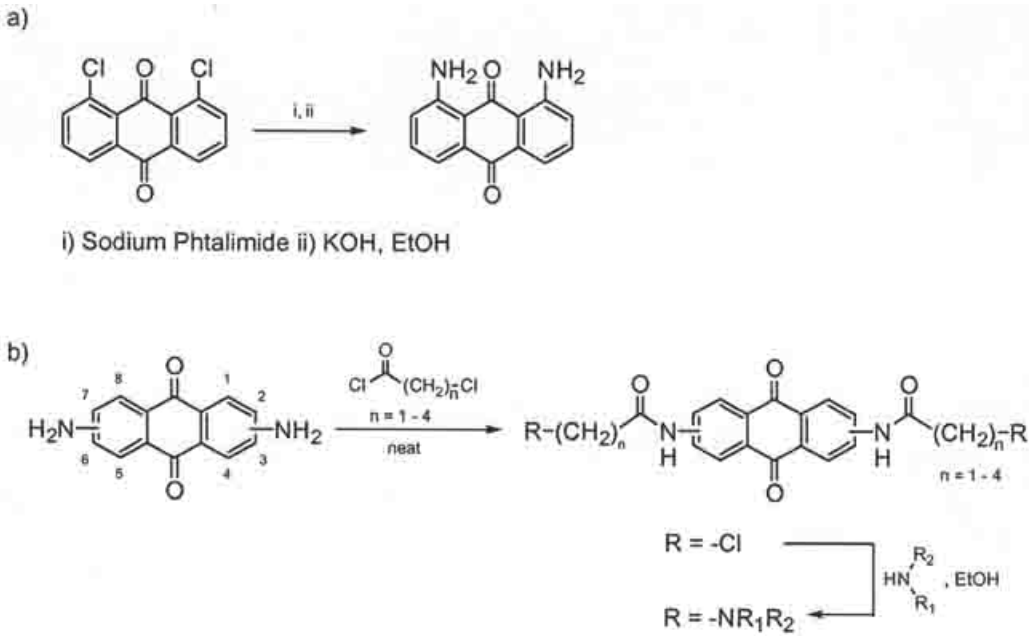

Fig. (3). Synthetic schemes for bis-aminoalkylamido anthraquinones.

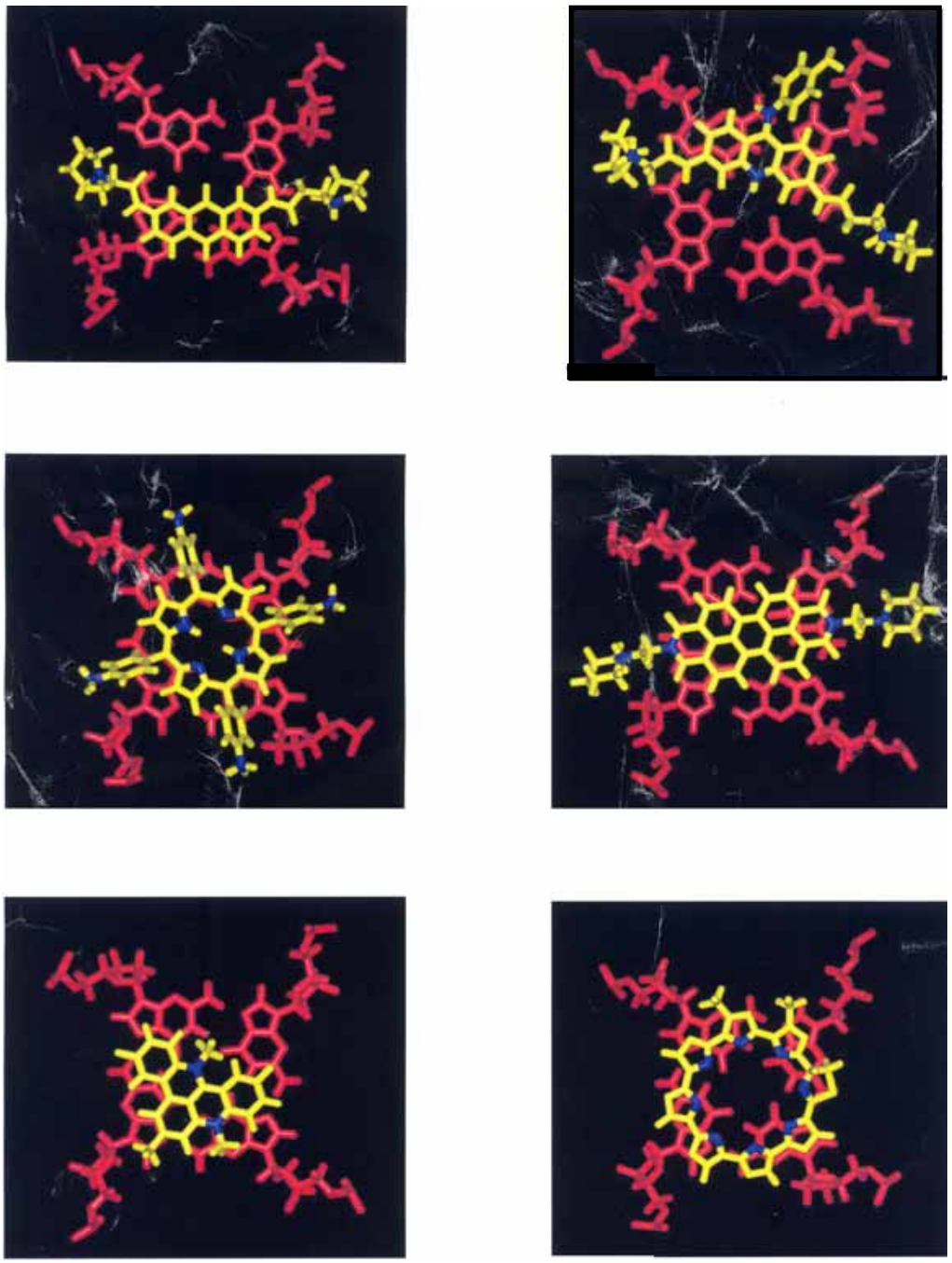

Fig. (4). Plots of low-energy minima for stacking of various G4 ligands onto the terminal G-quartet of the human quadruplex structure [20].
(a) A bis-aminoalkylamido anthraquinone [42].
(b) A 3,6,9-trisubstituted acridine derivative [44].
(c) TMPy4 [50, 51].
(d) PIPER [55].
(e) RHSP4 [62, 63].
(f) Telomestatin [65].

improved activity compared to the analogous 2,7-bis-amidoanthraquinones. This can be ascribed to the poor electron-donating properties of the amido groups, resulting in the ring nitrogen atom being only weakly basic. Thus the chromophore has electronic characteristics analogous to the amidoanthraquinone moiety. The synthesis of this type of compound starts from the commercially-available and inexpensive 3,6-diaminoacridine derivative proflavine and

Table 1. Telomerase Inhibition of Disubstituted Amido-anthraquinones (AQ), Fluorenones (FL) and Acridines (AC). EC_{50} Values are in μM. Data are From [39-41]

Substituent	1,4 AQ	1,5 AQ	1,8 AQ	2,6 AQ	2,7 AQ	FL	3,6 ACI
Diethylamino	1.8	2.7	4.2	3.5	4.3	15.5	5.8
Dimethylamino	nd	1.3	6.4	4.1	4.7	16.2	8.2
4-Morpholino	33.5	>50	>50	>50	>50	>50	>50
1-Piperidino	nd	2.3	3.7	4.5	3.1	9.0	2.8
1-Pyrrolidino	nd	nd	nd	1.8	2.0	nd	5.2

follows the same general route as for the compounds described above.

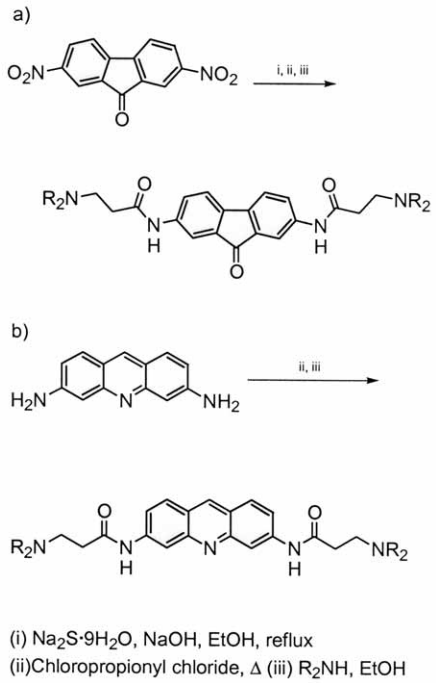

Fig. (5). Synthetic schemes for bis-aminoalkylamido fluorenones.

The problem of ligand selectivity for G-quadruplex over duplex DNA has been approached by means of structure-based design methods [44]. In general, quadruplexes have four phosphodiester strands forming four separate grooves in the structure, compared to the two in B-form duplex DNA [45]. Previous modelling studies have suggested that the two substituents of the bis-amido tricyclic chromophores each reside in a quadruplex groove. A third substituent, an anilino group at the 9-position on the acridine chromophore, fits into a third groove in this model (Figure **4b**). The 9-substituent also enhances the basicity of the acridine central ring nitrogen atom. A number of 3,6,9-trisubstituted acridines have been synthesised [45] via acridone intermediates as shown in Figure **6**. The increase in telomerase potency for these compounds in the TRAP assay (Table 3) compared to the disubstituted analogues, correlates with increased quadruplex binding affinity as measured by SPR, and provides further support for the theory of G-quadruplex mediation in the inhibition of telomerase activity. More recently-designed 3,6,9 tri-substituted acridines have telomerase potencies in the 10-20nM range [46], which is > 1000-fold their IC_{50} values. This suggests that sub-nM telomerase inhibition together with >25 μM cytostatic activity is an attainable goal. The earlier compounds in this series [45] also cause senescence in long-term cell culture at sub-cytotoxic doses, and show *in vivo* activity against tumour xenografts [47].

Bis-intercalation is well-established for duplex DNA, whereby two linked chromophores simultaneously interact with a pair of sites. This principle has recently been extended to the evaluation of a cyclic macrocycle with two acridine moieties linked by two 2,7-substituted diethylenetriamine side-chains [48]. This molecule is an effective quadruplex stabiliser, with FRET methods showing

Table 2. Cell Growth Inhibition (IC_{50} Values in μM) for Disubstituted Amidoanthraquinones (AQ) and Fluorenones (FL) in the A2780 Ovarian Carcinoma Cell Line. Data are from [39-41]

Substituent	1,4 AQ	1,5 AQ	1,8 AQ	2,6 AQ	2,7 AQ	FL	3,6 ACI
Diethylamino	0.2	0.32	0.64	2.35	2.1	12	0.57
Dimethylamino	0.02	0.35	0.33	2.55	2.1	14	0.75
4-Morpholino	3.7	>25	>25	>25	5.3	>25	3.1
1-Piperidino	0.29	0.43	0.54	1.3	0.48	11	1.7
1-Pyrrolidino	0.0025	0.32	0.29	39	1.2	nd	2.65

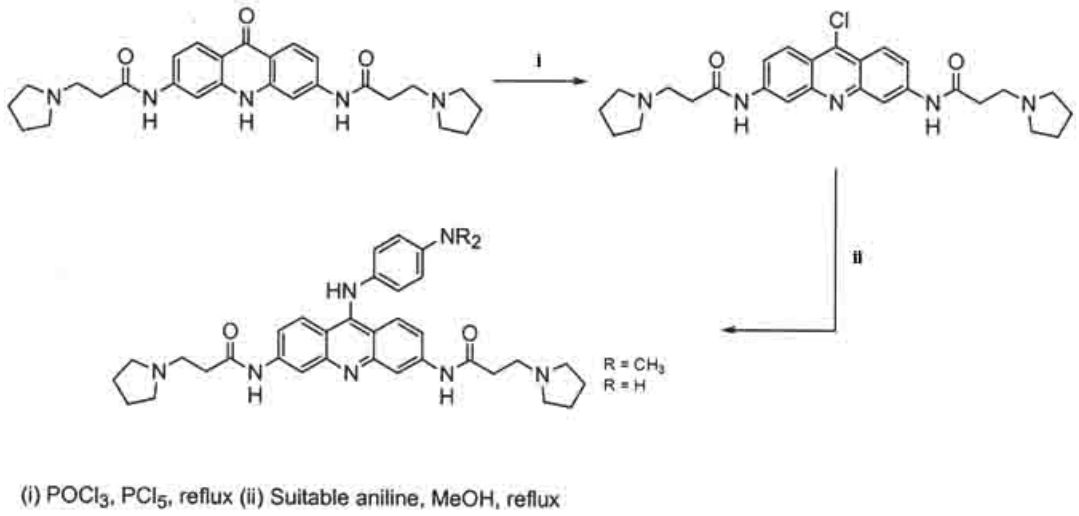

Fig. (6). Synthetic schemes for 3,6,9-trisubstituted acridine derivatives.

a ΔT_m of 15° compared to 0° for the acridine monomer. It is a moderately potent telomerase inhibitor, with an EC_{50} value in the TRAP assay of 0.75μM. No structural or modelling data has been reported on the nature of the dimer binding to the human intramolecular quadruplex. The maximum possible separation for the two acridine chromophores is ca 10.2 Å, which is probably too short, by 3.4 Å, for simultaneous interaction with both ends of the stack of three G-quartets.

Table 3. Telomerase Inhibition (EC_{50}) and Cell Growth Inhibition (IC_{50}, in the A2780 Ovarian Carcinoma Cell Line) of Trisubstituted Amidoacridines, in μM. Data are from [44]

Substituent	EC_{50}	IC_{50}
H	5.2	2.65
N-Phe-$N(CH_3)_2$	0.095	10
N-Phe-NH_2	0.06	>25

Ethidium derivatives with a variety of substituents have been found to be potent telomerase inhibitors, with the extent of inhibition correlating well with binding to the human intramolecular G-quadruplex [49]. The most active compounds reported in this study, with cationic phenylamidinium substituents, have been previously been reported as anti-trypanocidal drugs. Their telomerase EC_{50} values are reported to be in the 18-20 nM range, compared to that for ethidium itself, of 0.2-0.3 μM. (The modified TRAP assay used in our laboratories [39, 40] gives a value of ca 3 μM for ethidium).

POLYCYCLIC G4 LIGANDS

A number of tetrapyridyl-substituted porphyrins have been found to be effective telomerase inhibitors at the low μM level [50, 51], and bind strongly to several types of quadruplex structure. Structure-activity relationships have been developed using molecular modelling [32, 50], and both telomerase inhibition and quadruplex binding depend strongly on the nature of the substitution [50, 52]. Modelling studies suggest that the four-fold symmetry of these compounds enables them to interact effectively with the G-quartet arrangement (Figure **4c**), with the substituents interacting in all four grooves. Surprisingly, there is a low extent of pyrrole ring overlap with guanines. Antitumour activity has been reported for the lead compound 4-N-methyl-tetrapyridyl-porphin (Figure **2**), with observations of chomosomal end-to-end fusions providing good presumptive evidence of telomeres being the target for these compounds [53, 54].

The polycyclic compound PIPER (Figure **2**), based on the perylene skeleton, is an effective telomerase inhibitor, in the low μM range, that was devised by use of an automated *in silico* procedure to find an optimally-sized chromophore [55] for interaction with quadruplexes. An NMR model of quadruplex binding suggests that it binds to quadruplexes strongly, at G-quartet ends [54], and it acts in a chaperone-like manner, accelerating the folding process [28]. Selectivity for quadruplexes over duplex DNA has been reported to be favoured, by up to 10^3 for PIPER and several analogues under conditions (of pH ca 8.5) where PIPER is aggregated [56]; at physiological pH, selectivity is only 10-fold. PIPER has also been shown to stabilise both the G-rich [57] and complementary C-rich strands [58] in a sequence from the c-*myc* promoter. DNA cleavage activity has been appended to the PIPER molecule with the synthesis of an EDTA-Fe^{2+} complex [59]. This compound is selective for quadruplexes, and appears to bind in the same manner as PIPER itself, ie on the exterior of a G-quartet stack.

Increasing the size and therefore the accessible planar area of a chromophore from three to four or more fused rings does not necessarily result in enhanced G-quartet overlap, largely since the guanine bases are arranged rather more around the perimeter of the quartet. Two tetracyclic systems have been reported, both with only moderate telomerase inhibitory activity and EC_{50} values, of 7 and 16 μM

respectively [60, 61]. Modelling shows only low overlap with guanines in the G-quartet. Even the polycyclic system of PIPER, with seven fused six-membered rings, has less activity against telomerase than might initially be expected (Figure **4d**). This is explained by the minimal overlap of PIPER with two of the four guanines in the terminal G-quartet of a quadruplex. The inclusion of a positive charge in the polycyclic system has a significant effect on activity, as has been found in the trisubstituted acridine series described above. The pentacyclic methylacridinium compound RHPS4 (Figure **2**), with a cationic nitrogen atom at the centre of the ring system, shows selectivity for quadruplex over duplex and single-stranded DNA [32], and potent telomerase inhibition, with an EC_{50} of 330 nM [62, 63]. Its ability to inhibit cell growth by cytotoxic mechanisms is only moderate, with IC_{50} values ranging between 0.5 and 18 μM in a range of tumour cell lines. RHPS4 inhibits cell growth at concentrations significant below that for acute cytotoxicity, and produces reduced expression of the telomerase catalytic sub-unit hTERT. This compound stacks onto a G-quartet such that the cationic charge is positioned above the channel of negative electrostatic potential in the quadruplex (Figure **4e**), thus enhancing overall interaction. The structural model from the NMR study [32] is in striking agreement with this predicted geometry for the complex.

The quadruplex interaction of a series of pentacyclic dibenzophenanthroline ligands with crescent shaped arrangements and extended side-chains have been studied by FRET methods [64]. The series shows a high correlation between extent of quadruplex binding and telomerase inhibition, with the most active compound having an EC_{50} of 28 nM. The remarkable natural product telomestatin (Figure **2**), from *Streptomyces anulatus*, has been reported to have an EC_{50} value of 5 nm [65]. Although no comparative TRAP assay data have been reported so that telomostatin can be fully compared with other inhibitors, its predicted high affinity for a G-quartet structure (Figure **4f**), suggests that it is among the most active telomerase inhibitors known.

CONCLUSIONS

The number and diversity of G-quadruplex ligands has grown rapidly since the first demonstration of activity in 1997 [14]. The hypothesis that their ability to inhibit telomerase is a consequence of quadruplex binding, has received extensive support from a number of studies. It is highly likely that sub-nanomolar enzyme inhibition is achievable in the near future, either as a result of combinatorial or rational design approaches. The concomitant need to minimise short-term acute cytotoxicity, so that inhibitors are both potent and cytostatic, may be best achieved by rational design means. The recent crystal structure of the human intramolecular quadruplex [20] may help to attain this goal, although structural data on ligand complexes will be of yet more value.

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