DNA Tetraplex-Binding Drugs: Structure-Selective Targeting is Critical for Antitumour Telomerase Inhibition

Philip J. Perry and Terence C. Jenkins*

Yorkshire Cancer Research Laboratory of Drug Design, University of Bradford, Bradford, West Yorkshire BD7 1DP, U.K.

Abstract: Four-stranded tetraplex ("G-quadruplex") DNA represents a new paradigm for the design of DNA-interactive antitumour drugs, as the formed DNA–drug complexes have been suggested to interfere with critical telomerase function. The unique structural features presented by tetraplex over duplex DNA have stimulated the design of small ligand molecules able to selectively promote the formation and/or stabilisation of such higher-order DNA structures. Current developments in tetraplex-targeted telomerase inhibitors, and importantly their DNA structural selectivity, are explored.

INTRODUCTION

The discovery and development of small molecules capable of binding to nucleic acid biotargets, typically double-stranded DNA or hybrid DNA–RNA duplexes, continues to be a rewarding area for new antitumour chemotherapeutic agents. In large part, this view is reinforced by information gained during the past 30 years from studies with many current clinical anti-cancer agents (e.g., anthracyclines, cisplatin, alkylating agents, etc.), where DNA interaction or reactivity is often implicated in their overall potency. Further, our rapidly expanding understanding of the fundamental processes involved in the malignant transformation of cells allows a wider appreciation of the key molecular events that can potentially be intercepted at the nucleic acid level using drug-based strategies. Thus, for example, the targeting of individual base sequences and the subsequent down-regulation or prevention of gene expression by engineered drugs offers a potential for selective therapeutic targeting of genetic diseases, including cancer. Increasingly, the effective design of such agents will also provide a valuable arsenal of diagnostic tools for use in gene target validation and functional genomics [1,2].

Many classes of synthetic and naturally occurring low molecular weight agents are known that interact with DNA through a variety of distinct mechanisms, including non-covalent (reversible) or covalent fixation processes. Aspects of the processes involved are beyond the scope of this review, but have been described elsewhere [1]. However, most drug-based strategies have exploited the antigene approach, where double-stranded DNA is targeted directly by a ligand molecule so as to interfere with template transcription function or replicative processes. Binding mechanisms typically involve either interaction in the minor or major grooves of the host duplex, or intercalation between stacked base pairs, although mixed-mode binding is also often evident (e.g., ref [3]). Biological response is primarily governed by the effective residence time of a bound molecule with cytotoxic effects arising from cellular events that require the unimpeded DNA template. To date, major effort has largely focused on the design of improved duplex-interactive molecules in order to maximise biological potency using conventional medicinal chemistry approaches. A broad spectrum of DNA-interactive compounds has now been evaluated for clinical application using principles that stem from this design method.

The finding that, under certain circumstances, a third DNA strand can bind within the major groove of duplex DNA to form a high-order triplex structure aroused the development of triplex-forming oligonucleotides for use in parallel therapeutic strategies [2,4–7]. Antigene approaches of this type can potentially target genomic sequences to modulate their expression or interactions with DNA-binding proteins, interfere with template function, or provide molecular biology tools. In principle, many diseases including cancer can thus be treated by targeting defined genes of suitable double-stranded sequence with either an administered or vector-delivered triplex-forming oligonucleotide to generate a local DNA triplex. Successful inhibition of transcription has been achieved for selected target genes using this strategy [5–10].

Unfortunately, DNA triplexes are characterised by rather poor thermodynamic stability and this factor has posed practical obstacles for effective biological application [11–14]. To this end, a new class of ligand was sought to differentially improve triplex stability and/or promote *in situ* triplex formation, using methodologies developed for lower-order DNA duplex-directed agents. Aspects of the successful drug themes for "antigene enhancers", involving intercalation, groove-binding and charge neutralisation mechanisms have been reviewed [4,6,7]. Such studies represent the first developments in the structure-selective

*Address correspondence to this author at the YCR Laboratory of Drug Design, University of Bradford, Bradford, West Yorkshire BD7 1DP, U.K. Telephone: [+044] (0)1274 236480; Fax: [+044] (0)1274 236481; e-mails: p.j.perry@brad.ac.uk or t.c.jenkins@brad.ac.uk

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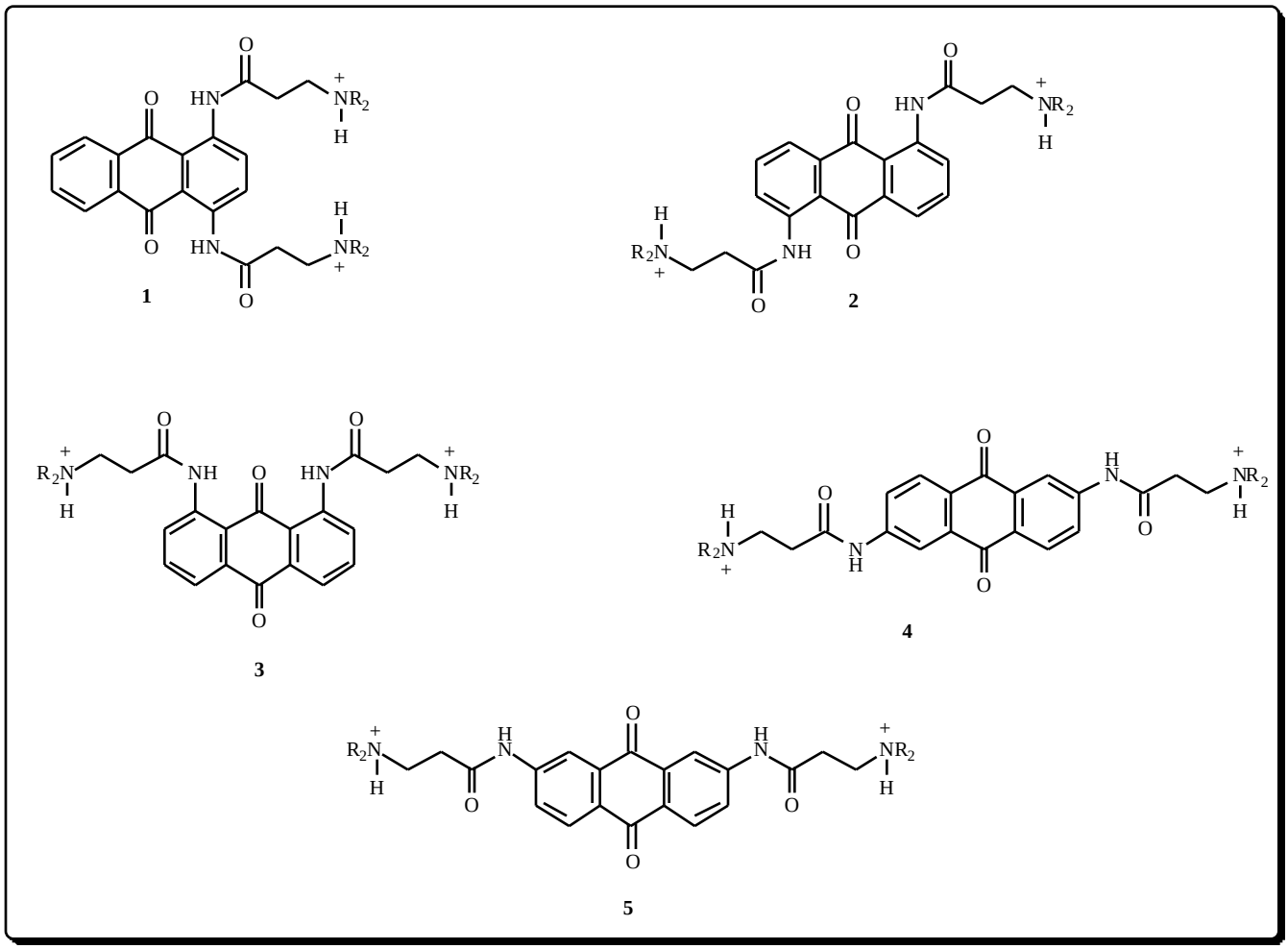


targeting of high-order multi-stranded nucleic acids by a tailored ligand. Developments in this area are illustrated by difunctionalised anthracene-9,10-diones ("anthraquinones, AQ", e.g., **1**–**5**), where the DNA triplex stabilisation and biological profile (cytotoxicity, mutagenicity, bioavailability, etc.) can be manipulated to improve drug efficacy [2,4,15–20].

Elegant studies of the transformation of normal human cells to tumour cells have established an intimate link between tumour immortalisation and activity by the enzyme telomerase [21,22]. This enzyme serves to maintain the length of telomeres, the specialised DNA sequences at the 3'-end of chromosomes that comprise tandem 5'-TTAGGG repeats in humans, and is found in some 85–90% of human tumours but invariably absent in somatic cells. Telomerase has thus emerged as an obligatory anti-cancer chemotherapeutic target [23–28], and considerable effort is now focused on the design of agents with inhibitory activity (reviewed in refs. [23,24]). Documented reports that G-rich DNA sequences of this type can assemble to four-stranded tetraplex (or "G-quadruplex") structures, as detailed below, suggested that such structures could be targeted to effect an indirect inhibition of the enzyme [2,23,24,29–31]. This idea stimulated a renewal of interest in the design of DNA-interactive drugs that can selectively recognise, bind and divert the telomerase substrate behaviour of telomeres in whole cancer cells. Recent developments in DNA tetraplex-binding drugs for antitumour telomerase inhibition are discussed in this review.

Interestingly, stable DNA systems of even higher 6-fold order have been demonstrated where a dimeric structure is obtained by stacked assembly of two DNA tetraplexes through a hexad interface that involves six DNA bases on each of the two molecular faces [32]. Analogous DNA sequences are implicated in certain immunoglobulin receptor genes and also in cellular DNA segments that can cross-hybridise with viral regions. This comparatively recent explosion in structural and biochemical information suggests that the significance of high-order DNA structures with three, four, six (or more) strands is only now emerging. Similarly, the potential for drug-based therapeutic intervention at this level is slowly being recognised.

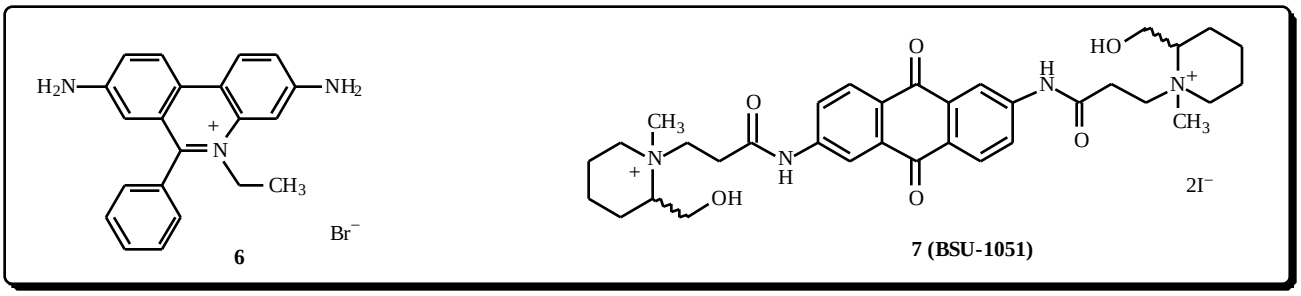
BIOLOGICAL ROLE OF DNA TETRAPLEXES

Guanine-rich DNA sequences can assemble or fold to generate G-tetraplex structures in solution at physiological concentrations of Na^+ and K^+, although their formation has yet to be established *in vivo*. However, despite this lack of detection *in vivo*, such structures are believed to play a vital cellular role; further, the conservation of chromosomal telomeric DNA sequences may be related to their inherent tetraplex formation [33]. This hypothesis is supported by

findings that many telomeric DNA-binding proteins bind to tetraplexes and/or promote their formation [34–40], and may also regulate telomerase activity [41,42]. In addition, a number of helicases capable of unwinding G-tetraplex DNA have recently been reported [43–46], providing further circumstantial evidence to support a cellular role for such high-order DNA structures.

G-TETRAPLEX-INTERACTIVE LIGANDS

All DNA tetraplexes require stabilisation by monovalent metal ions, where the $K^+ > Na^+$ rank order found stems from a superior fit to the cavities presented by the G-tetrad planes; the assembly or folding of G-rich DNA strands is thus favoured under high-[K^+] salt conditions [47–49]. Under these conditions, telomerase processivity is inhibited as the enzyme is prevented from necessary access to its linear DNA substrate [50–53]. In large part, this activity has directly prompted the current quest for small-molecule ligands as telomerase inhibitors that can selectively stabilise DNA tetraplexes by mirroring the behaviour of K^+ ions [23–26].



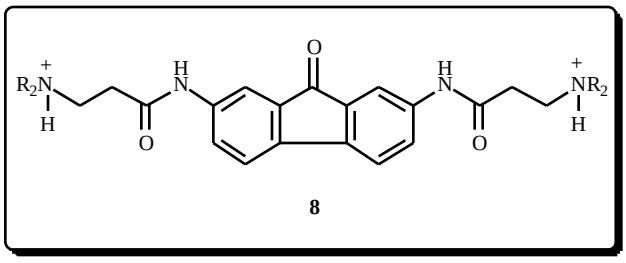
Suitable agents would find versatile application in telomerase assay methods, and as DNA-directed gene probes for tetraplex formation [24].

The development of agents for the structure-specific recognition of DNA tetraplexes to effect *in situ* cellular inhibition of telomerase function has largely stemmed from experience with intercalators for duplex or triplex DNA. Thus, most compounds to date are based upon planar extended-aromatic ligands where DNA binding is anticipated via molecular π-overlap with the G-tetrad planes and with any attached side-chains and/or pendant charge centres serving to anchor the bound drug to the polyanionic DNA host. Given the successful development of modified anthraquinones ("AQ"; e.g., **1**–**5**) as triplex-specific ligands [15–19], together with structural insight from DNA duplexes with mismatched G·G base pairings [54,55], it was suggested in 1993 that these or similar agents may also behave as tetraplex stabilants [56]. Qualitative support for this strategy was aroused by a report that the fused-ring ethidium bromide (**6**) dye binds to an intermolecular DNA tetraplex structure (i.e., $[d(T_4G_4)]_4$) through a mode that is consistent with intercalation of the G-tetrad planes [47].

Qualitative molecular models constructed for DNA tetraplex–AQ complexes based upon a "threading" intercalation binding model confirmed that tetrad–drug π-overlap was feasible and that any flexible pendant side-chains could be accommodated in the wider tetraplex grooves. This resulted in the first report of a non-nucleoside tetraplex-interactive inhibitor of human telomerase, with a 2,6-difunctionalised anthraquinone derivative (BSU-1051, **7**) displaying a telomerase inhibitory value ($^{tel}IC_{50}$) of 23 μM determined using a 5'-TTAGGG primer extension assay [57]. Importantly, this study also demonstrated involvement of an intermolecular tetraplex–ligand complex as the stalling of telomeric elongation appeared with a periodicity corresponding to four repeat sub-units. Confirmatory support for this model was provided by enhancement of DNA polymerase arrest in the presence of K^+ ions [53].

We have subsequently reported the telomerase inhibitory activities of >50 related anthraquinone derivatives, including five distinct regioisomeric series (e.g., structures **1**–**5**), in an attempt to probe structure–activity relationships [58,59]. Telomerase inhibitory values ($^{tel}IC_{50}$) in the 1–50 μM range were found using a modified version of the universal TRAP assay, although no obvious structure–activity relationship could be discerned for these geometric isomers. Additionally, isothermal titration calorimetry (ITC) was used to examine the thermodynamic binding properties of selected ligands with a portion of the intramolecularly folded human telomeric DNA tetraplex (i.e., $d[AG_3(T_2AG_3)_3]$), showing only weak binding affinity of only ~10^4 M^{-1} [59]. Interestingly, the most active compounds from this series ($^{tel}IC_{50}$ values of 1–5 μM) rank as the most potent small-molecule inhibitors of human telomerase reported to date.

Telomerase inhibition has since been reported for other tricyclic chromophore systems intended to be near-isostructural with 2,7-difunctionalised anthraquinones (**5**), including a series of fluorenone analogues (**8**) designed to ameliorate cytotoxicity by prevention of redox cycling through removal of one of the quinone carbonyl moieties [60]. The most potent compounds in this series displayed $^{tel}IC_{50}$ values of 8–12 μM, with a typical 2–10-fold reduction of conventional cytotoxicity to a panel of human tumour-derived cell lines compared to their equivalent anthraquinone analogues. However, no evidence for interaction with DNA tetraplexes was presented.



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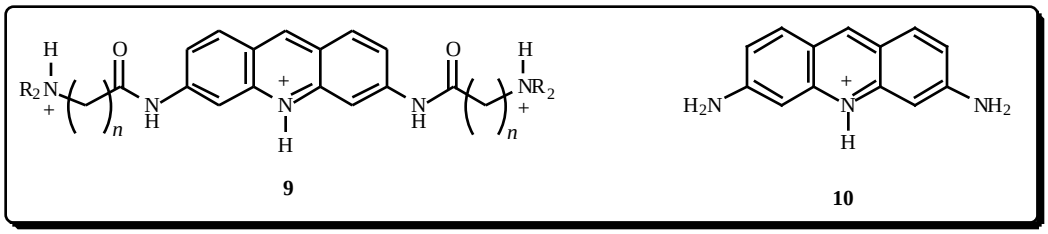

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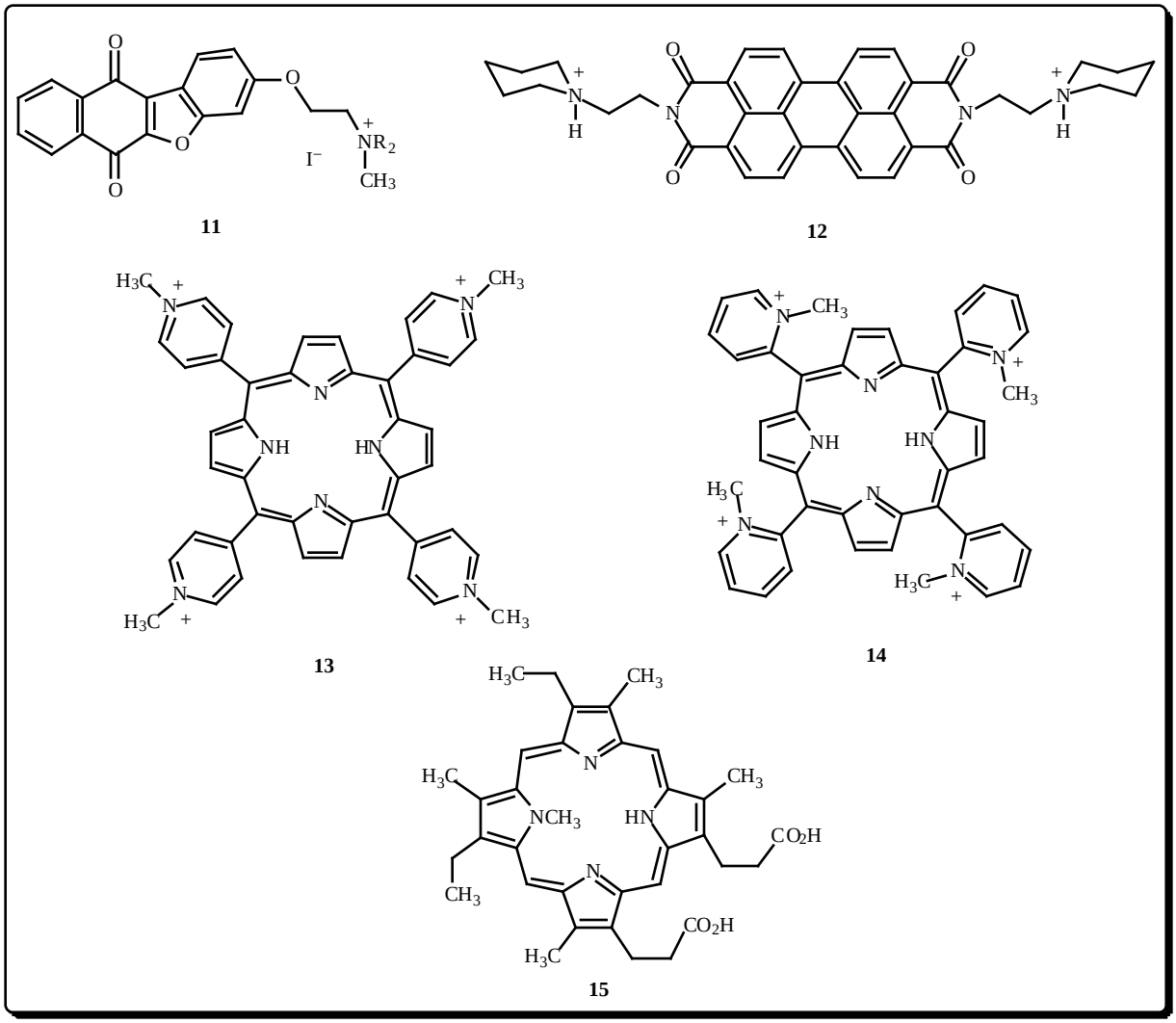
Telomerase inhibition by a number of functionalised proflavines (**9**), first proposed and synthesised by one of us [2] has similarly been reported ($^{tel}IC_{50}$ = 1–50 μM) by others [61], although these molecules appear to offer little advantage over the simpler parent acridinium species. Indeed, the inhibitory potency of proflavine (**10**) is remarkably similar ($^{tel}IC_{50}$ = 3.9±0.4 μM; TCJ, unpublished data) and suggests that elaboration of the side-chains is ineffective and not required for this molecular skeleton. Moreover, degradation studies indicate that the functionalised analogues (**9**) may be unstable due to autocatalytic hydrolysis to proflavine (**10**) under the enzymatic conditions employed for the TRAP assay.

A number of compounds containing "extended" planar aromatic π-systems that interact with G-tetraplex DNA and inhibit telomerase have recently been described. These include two tetracyclic quinone derivatives (**11**) with $^{tel}IC_{50}$ values of 5.4 and 7.0 μM [23,62], a larger perylene-based diimide (**12**; $^{tel}IC_{50}$ ≈ 40 μM, [63]) capable of accelerating G-tetraplex formation [64] and inhibiting G-tetraplex unwinding by *Sgs*1 helicase [65], and a number of porphyrin derivatives (**13**–**15**) intended to maximise aromatic π-overlap with a host G-tetrad. Porphyrins can also catalyse the interconversion of DNA tetraplexes related to telomeres [66], although single-stranded DNA sequences have also recently been shown to have a similar influence [67].

The cationic tetra-(*N*-methyl-4-pyridyl)porphine (**13**) was recently shown to bind to tetraplex DNA [68–72] and inhibit telomerase activity, with a reported IC_{50} value of 6.5 μM using a cell-free primer-extension assay that avoids a PCR-based amplification stage [68]. However, while such inhibition was shown to be dose- and time-dependent, no



11 **12** **13** **14** **15**

detectable effect on telomere length was evident. *In vitro* telomerase inhibitory activity by (**13**) has been demonstrated with intact MCF7 human breast carcinoma cells at subtoxic drug concentrations [73], and involvement of a chromosomal tetraplex has been established with an *in vivo* system [74]. A wide spectrum of related porphyrin derivatives with broad-ranging inhibitory activity has since been examined, providing a basis for preliminary structure–activity relationships. Metal-chelated derivatives were generally less active than the free porphyrins (**13**), and most naturally occurring porphyrins displayed only limited activity. Further derivatives were evaluated in an extended systematic search in order to explore (i) π-stacking ability, (ii) groove width, and (iii) possible H-bond and/or electrostatic interactions within the formed DNA tetraplex–drug complex. Interestingly, the *para*- and *ortho*-substituted isomeric porphyrins (**13**) and (**14**) showed contrasting inhibitory activity ($^{tel}IC_{50}$ = 6.5 and >65 μM, respectively), despite apparently similar affinities for a model human DNA tetraplex system [71]. The closely related mesoporphyrin dye NMM (**15**), expected to be essentially anionic rather than cationic in aqueous solution at physiological pH, was shown to favour binding to tetraplex structures rather than duplex-form DNA [70,76].

However, while it is established that these agents bind to tetraplex DNA and that this binding must be implicated in the telomerase inhibition, the mode(s) of binding are by no means unambiguous. For example, the stoichiometries, affinities and sites for binding remain controversial, with alternative intercalated, end-pasted or 'sandwich-type' interaction modes suggested for π-stacked stabilisation of the DNA–drug complexes (reviewed in [2]). This situation is confused by the diversity of DNA tetraplexes used in drug studies, where attention has focused on inappropriate blunt-ended or parallel-stranded intermolecular 'model' systems rather than more relevant intramolecularly folded biological structures. In the absence of definitive structural information, other than derived from NMR observations, the interactions of porphyrin (**13**) with three tetraplexes (including the model

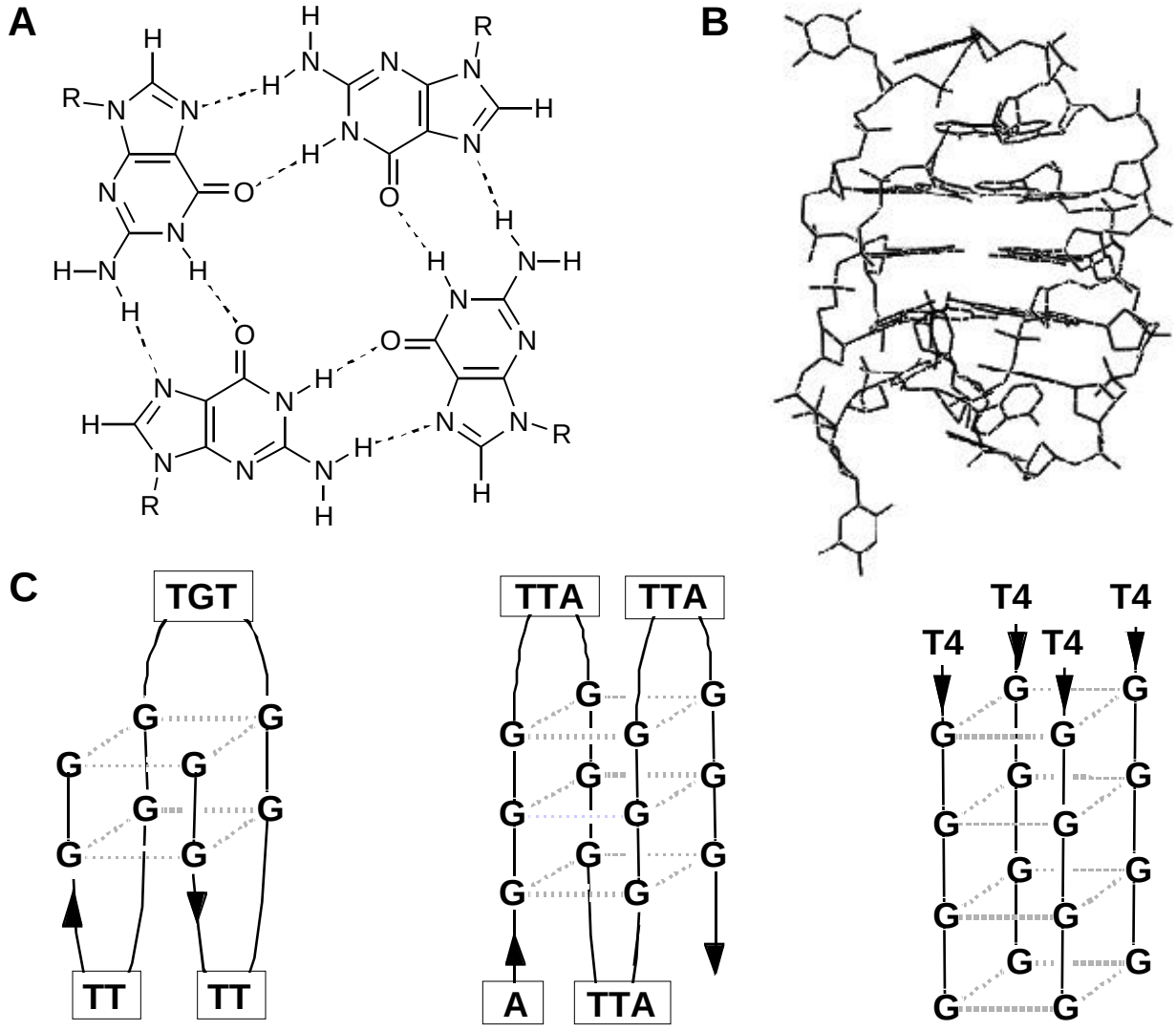


Fig. (1). Structures of (A) an individual guanine tetrad showing the hydrogen bonded square planar motif, and (B) the solution NMR structure determined for the folded human telomeric d[$AG_3(T_2AG_3)_3$] tetraplex [78]. The folding topologies for three commonly studied tetraplexes, G2 (15-mer), G3 (22-mer), and G4 (4 × 8-mer), respectively, with 5' → 3' strand alignment are represented schematically in (C).

human structure, Figure 1) were examined using a detailed thermodynamic (ITC), molecular modelling and spectrophotometric approach with solution conditions that favour the high-order host DNA structures [72]. This study confirmed that binding is actually rather weak ($0.3–2 \times 10^5$ M^{-1}), and that the stoichiometry is governed by the number of stacked G-tetrads in each DNA structure (e.g., 2 porphyrins are accommodated in the model human tetraplex with three G-tetrad planes). Further, the thermodynamic parameters were entirely consistent with drug intercalation rather than alternative end-pasted or "sandwich" binding mechanisms. Further studies are currently in progress to assess whether such a mode could be universal, but it is already clear that the binding behaviour is also influenced by the solution conditions and the DNA complexion. Importantly, caution is required in comparing binding properties from alternative or dissimilar assay methods at this stage.

There has been a marked paucity of structural support for any binding model using either solution NMR or X-ray crystallographic methods, with greater emphasis instead placed upon molecular modelling or indirect biochemical data (e.g., [75]). Such studies must be viewed as inconclusive, particularly given the inherent conformational flux and potential for structural heterogeneity in high-order DNA tetraplex systems (particularly intermolecular assemblies) under biologically relevant solution conditions [14,17,49]. Thus, for example, assumptions are often made concerning binding stoichiometry and a common distribution of sites in a given tetraplex so as to favour one particular model. However, the finding that the common 2:1 stoichiometry established for binding either ethidium bromide (**6**) or porphyrin (**13**) to the folded human telomeric tetraplex is reduced to a 1:1 ratio for selected anthraquinones (albeit without saturation of the host tetraplex at the concentrations used in ITC experiments) suggests that binding is dictated by steric accommodation factors. Detailed structural studies are required to establish where the ligand molecules are actually located within a given DNA–drug complex, and it seems unlikely that one universal binding model could be implicated for such a wide spectrum of ligands.

STRUCTURAL-SELECTIVITY OF TETRAPLEX-INTERACTIVE LIGANDS

Developments in this class of inhibitor have thus far been either largely serendipitous or a logical progression from comparative studies with lower-order duplex-form DNA [2,23]. However, the primary objective requires a measure of tetraplex-specific (or -preferential) discrimination to optimise efficacy and prevent unwanted depletion of ligand by competitor DNA target sites. Thus, for example, unwanted drug binding to double-stranded DNA could lead to adverse cellular effects and/or arbitrary toxicity in normal cells rather than the intended tumour target. In this regard, scant attention has thus far been directed to the design of genuinely selective novel agents that will recognise only the tetraplex biotarget [2,23].

A clear requirement is for a quantitative assay to determine the relative binding affinities for host tetraplex, triplex, duplex DNA and single-stranded (DNA/RNA) nucleic acids for a given candidate inhibitor ligand. Fortunately, a rapid technique has now been established for this exact purpose [76,77], using a thermodynamically rigorous competitive equilibrium dialysis method that exploits therapeutically sensible concentrations of agent. A less-extended version of this powerful assay was originally used to gauge the relative duplex/triplex DNA-binding properties of two isomeric anthraquinones (**1** and **4**, where $–NR_2$ = $–NMe_2$; [17]). In the full assay, solutions of different nucleic acid structures (of identical concentration) are dialysed simultaneously against a common solution of ligand using appropriately buffered conditions. After equilibration the amount of ligand bound to each DNA is measured by spectrophotometry. More ligand will naturally accumulate in the dialysis tube containing the structural form of highest binding affinity and, since all of the DNA samples are in equilibrium with the same free ligand concentration, the amount of ligand bound is directly proportional to the binding constant for each conformational form. Thus, comparison between the DNA samples gives a rapid and thermodynamically reliable indicator of structural selectivity for any given ligand.

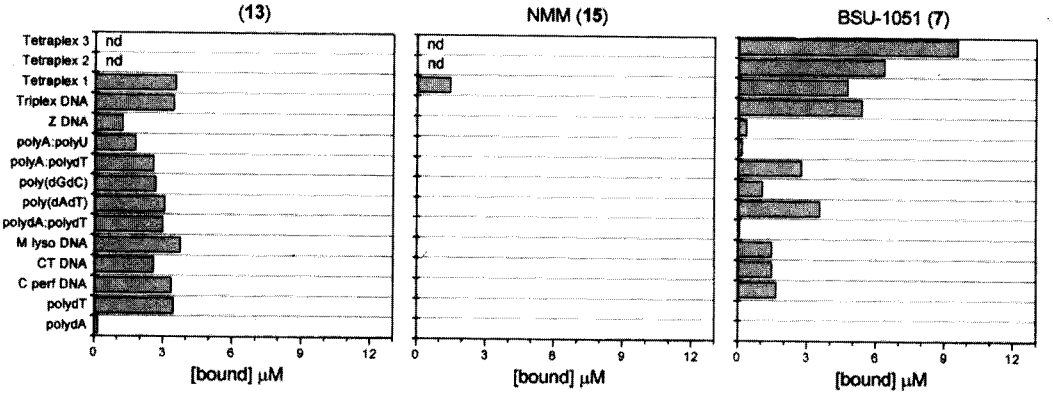


Fig. (2). Bar chart representation of results obtained by the competition dialysis method for three common tetraplex-interactive ligands, tetra-(*N*-methyl-4-pyridyl)porphine (**13**), the mesoporphyrin dye NMM (**15**), and anthraquinone BSU-1051 (**7**), respectively. The amount of ligand bound to each DNA structural form is plotted facilitating direct comparison of the structural preference(s) displayed by each ligand. nd = not determined.

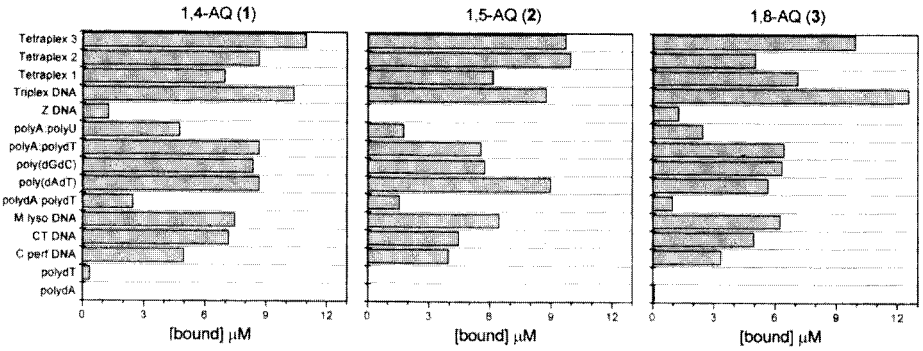


Fig. (3). Bar chart representation of DNA-ligand binding profiles for three regioisomeric anthraquinones (**1**, **2**, and **3**; $-NR_2 = -NMe_2$) determined by competition dialysis.

Using this technique, Ren and Chaires [76] recently examined the structural selectivity of various DNA-binding ligands, including the cationic porphyrin (**13**) and the structurally related mesoporphyrin dye NMM (**15**). The results obtained from competition dialysis experiments with each ligand are shown as bar charts in Figure 2, where the amount of ligand bound is plotted for each of the nucleic acid forms that were used. The porphyrin (**13**) binds in a uniformly weak and non-specific manner to all of the DNA structural forms that were investigated, including single-stranded (but *not* poly[dA]), duplex, triplex and tetraplex forms. In marked contrast to this evident promiscuous behaviour, the mesoporphyrin NMM dye (**15**) shows a clear structural selectivity for tetraplex DNA with no apparent affinity toward any other form of DNA. However, it is notable that the absolute level of binding appears to have been greatly compromised in this case to exact such a structural selectivity. We have now examined the behaviour of anthraquinone BSU-1051 (**7**) using an elaborated version of the assay procedure that uses two additional tetraplex DNA structural forms, including the human telomeric DNA tetraplex $d[AG_3(T_2AG_3)_3]$ (Tetraplex 2, [78]). The comparative results shown in Figure 2 [Ren, Perry, Chaires & Jenkins, unpublished] reveal that the 2,6-difunctionalised anthraquinone (**7**) displays a modest 2–5-fold preference towards higher-order (triplex and tetraplex) DNA forms over either duplex or single-stranded DNA. Disappointingly, this ligand offers only a 2–5-fold increase in affinity for the high-order DNA structures relative to the porphyrin analogues (**13** and **15**).

We have subsequently examined the binding profiles of a wide range of G-tetraplex-interactive anthraquinones (**1**–**5**) known to inhibit human telomerase activity [Ren, Perry, Chaires & Jenkins, unpublished]. Representative binding data are presented (Fig. 3) for three regioisomeric anthraquinones (**1**, **2**, and **3**; $-NR_2 = -NMe_2$), where these compounds exhibit outwardly similar binding profiles. In each case, relatively strong binding to tetraplex, triplex and duplex DNA structural forms is evident, where these behaviours reflect the reported telomerase inhibition [58,59], triplex-stabilising properties [15–17,20], and cytotoxic activities [59], respectively. However, the 1,4-disubstituted anthraquinone (**1**) is exceptional in that a misleading affinity is evident for triplex DNA. The apparently high binding affinity is not due to binding to the host triplex, but rather to the duplex that is released after ligand-induced dissociation of the third strand from the triplex [17]. This apparent anomaly serves to illustrate that an understanding of the underlying binding process(es) is required when interpreting data obtained from such a comparative assay

It is of paramount importance that undesirable systemic cytotoxicity is abolished, or at least minimised, for G-tetraplex-interactive inhibitors of telomerase to be effective [2,23,24]. We have attempted to address this issue with the

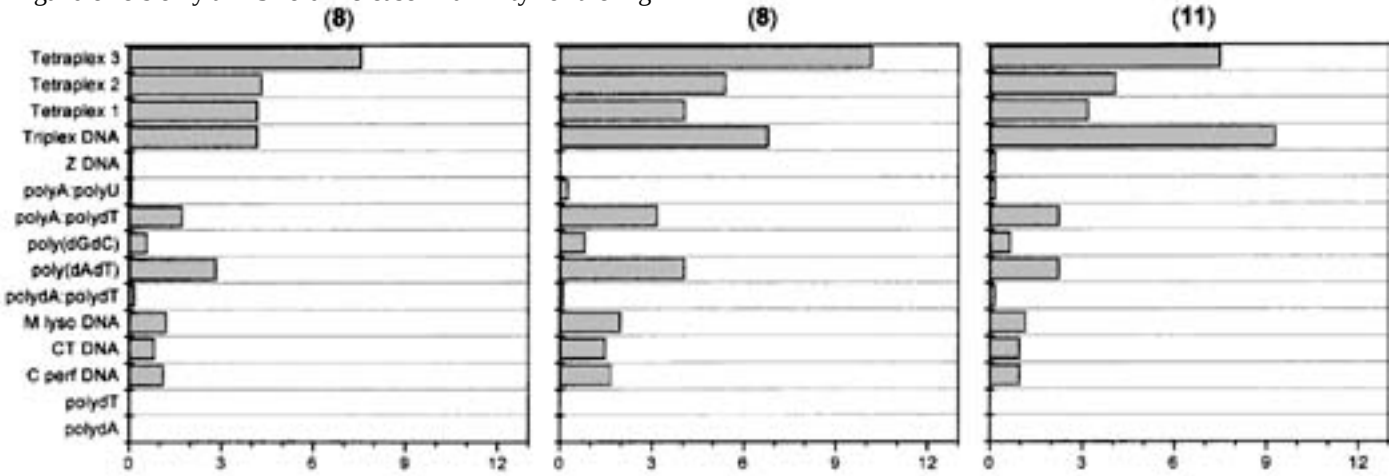


Fig. (4). Bar chart representation of DNA-ligand binding profiles for two fluorenone derivatives (**8**, $-NR_2 = -NMe_2$ and $-N^+Me_3$, respectively), and the tetracyclic quinone (**11**, $-NR_2 = -NMe_2$).

design of second-generation molecules, including a series of fluorenones (**8**) where the inherent redox-cycling potential of quinones is designed out by removal of one of the core carbonyl groups [60]. However, another significant contributory factor towards cytotoxicity for this class of molecule is binding to duplex DNA; hence, we have examined the DNA-binding profiles (Fig. 4) for a series of fluorenone-based telomerase inhibitors [Ren, Perry, Chaires & Jenkins, unpublished]. Figure (4) depicts the binding profiles of two representative fluorenone analogues (**8**, $-NR_2$ = $-NMe_2$ and $-N^+Me_3$, respectively) and the second-generation tetracyclic quinone (**11**, $-NR_2$ = $-NMe_2$). A clear reduction in binding affinity for duplex DNA relative to the anthraquinone molecules (**1**–**3**) is evident, and may be an additional contributory factor to the lower cytotoxicity displayed by these agents [60]. However, with the exception of Tetraplex 3, affinity towards higher-order DNA is also reduced, although a weak 2-fold binding preference over duplex or single-stranded DNA is evident. Similarly, the tetracyclic (**11**) displays a reduction in absolute levels of binding to duplex and single-stranded DNA relative to the anthraquinones (**1**–**3**), whilst retaining relatively tight binding to higher-order DNA structural forms. This finding highlights a clear structural preference for the curved monocationic molecule that contrasts with the behaviour shown by the linearly extended difunctionalised (i.e., anthraquinone, acridine or fluorenone) chromophores.

Read and co-workers have recently reported a molecular modelling investigation of the binding of a series of anthraquinone-based DNA tetraplex-interactive telomerase inhibitors (**4**) to the human G-tetraplex structure for a speculated binding site [79]. The authors claim an excellent agreement between predicted (computed) relative binding energies and telomerase inhibition for a limited range of compounds, with $^{tel}IC_{50}$ values in the 1 to >50 μM range. Despite this claim it is surprising that there appears to be only a poor correlation between inhibitory activity and binding behaviour determined under genuine thermodynamic conditions by ITC. On the basis of this claim, we have systematically examined the absolute binding affinities of an extensive selection of compounds from this series (**1–5**). For the first time, comparison is now made with reported biological data [58,59] to probe possible structure–activity linkages [Ren, Perry, Chaires & Jenkins, unpublished]. Binding data were determined using the dialysis method outlined earlier [76], as this assay was specifically designed to facilitate a direct comparison of relative DNA-binding affinities. Figure (5a) shows a scatter plot of *in vitro* cytotoxicity (IC_{50} values determined for human CH1 cells) versus absolute binding to double-stranded calf thymus DNA for a series of isomeric anthraquinones (**1–5**). Using a linear fitting procedure, a statistically significant fit is obtained ($p < 0.0001$, $n = 29$) that agrees with earlier suggestions that *in vitro* cytotoxic potency and duplex-binding affinity are positively correlated (e.g., refs. [80,81]). Similar correlations are obtained for other tumour-derived cell lines. Equivalent absolute binding data obtained for these compounds with a repeat portion of the human telomeric DNA tetraplex (Tetraplex 2) are shown in Figure (5b), where comparison is made with the reported telomerase inhibitory activities. In contrast to the reported claim of Read *et al*. [79], a satisfactory fit cannot be obtained for the available data ($p = 0.04$, $n = 28$) and there is no evidence of a statistically significant correlation between tetraplex binding and telomerase inhibitory activity. However, a possible correlation cannot be entirely dismissed given that the narrow range of data examined both by us and Read *et al*. [79] spans only a single order of magnitude in terms of activity. Nevertheless, given the available information, we must conclude that the suggested correlation between DNA tetraplex binding and telomerase inhibitory activity appears to be unfounded.

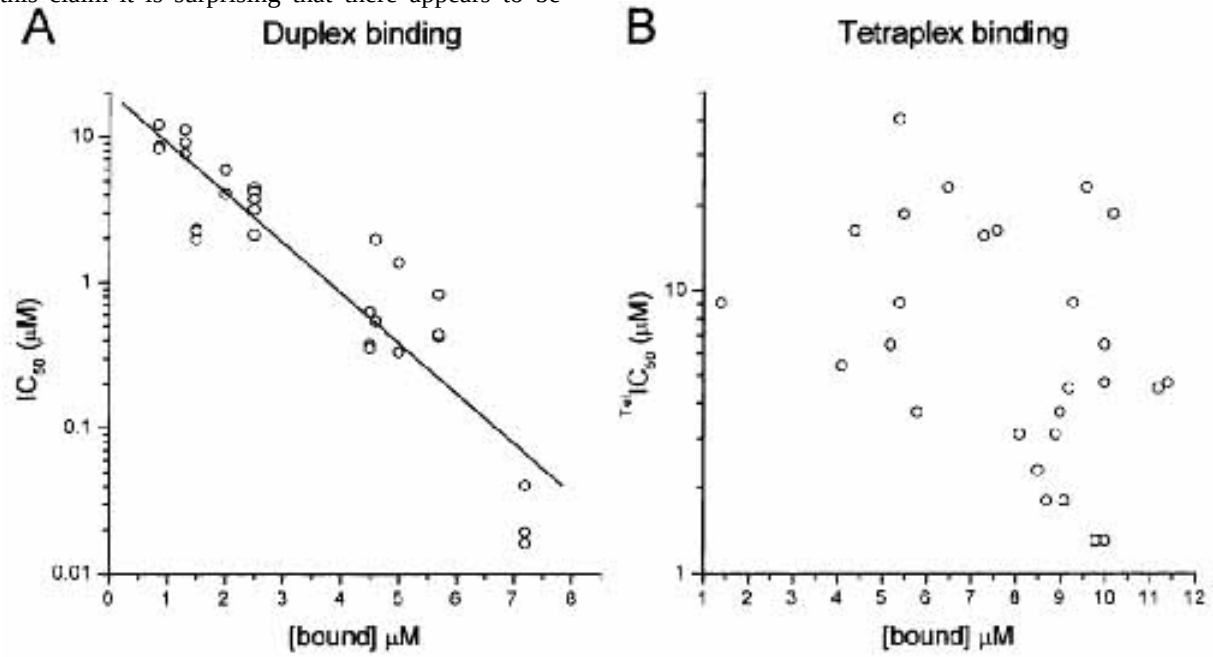


Fig. (5). Scatter plots representing DNA-binding determined by competition dialysis versus biological effect for a series of isomeric anthraquinones (**1–5**). (A) Correlation between *in vitro* cytotoxicity (for human CH1 cells) and binding to double-stranded calf thymus DNA ($p < 0.0001$, $n = 29$). (B) Scatter plot of binding to the human telomeric DNA tetraplex (d[$AG_3(T_2AG_3)_3$], Tetraplex 2) versus telomerase inhibitory activities, where a satisfactory fit cannot be obtained for the available data ($p = 0.04$, $n = 28$).

CONCLUSIONS

It is clear that our current knowledge of DNA tetraplexes is limited, particularly in terms of their inherent stability and/or potential for structural interconversion under physiologically relevant conditions. However, such detailed information will be vital for the design of agents that can selectively recognise and thereby promote or stabilise the formation of high-order DNA structures. While the existence of four-stranded DNA structures has yet to be positively demonstrated for intact biological DNA sequences, despite effort in many laboratories, it is probable that such structures do contribute to cellular replicative processes and are implicated in dynamic events involving condensed nucleic acids.

The demonstration that elevated K^+ levels can effect an inhibition of telomerase enzyme processivity, at least *in vitro*, has largely prompted the quest for effective K^+-mimics that can similarly stabilise tetraplex DNA structures, particularly the folded human d[$AG_3(T_2AG_3)_3$] structure, without the biological toxicity associated with K^+ ions. In this respect, both anthraquinones and porphyrins have been shown to be effective replacements, although their inherent binding affinities toward the DNA (K_b = 10^3–10^5 M^{-1}) are frustratingly poor. Such weak binding implies that enzyme access to the linear or unfolded DNA is unlikely to be prevented at the cellular or nuclear levels that can realistically be achieved with non-toxic levels of these therapeutic agents. On this basis, the earlier suggestion of a structure-selective binding and hence structural 'locking' of tetraplex DNA [56–59], whilst it may indeed contribute, does not appear to fully account for the observed enzyme inhibitory effect. The telomere DNA–protein recognition event required for enzyme processivity probably involves a >10^4-fold stronger binding than could be achieved with the present generation of tetraplex-interactive ligands. Thus, accumulation of the ligand (drug) in a folded DNA tetraplex would be expected to merely impede but *not* prevent access to the linear DNA substrate form. However, it is possible that further kinetic (e.g. structural or conformational reorganisation) rather than thermodynamic factors may play a direct role in the cellular context.

While our present levels of information are incomplete, it is here suggested that the biological efficacy of DNA tetraplex-targeted inhibitors of telomerase may instead arise from a combination of competitive binding events involving DNA and/or hybrid DNA–RNA structures, or derived ternary DNA–enzyme–drug complexes, where the underlying target nucleic acid structures are of lower strand order. Thus, for example, we find that DNA tetraplex binding is negligibly influenced by the geometric pattern of ring substitution for anthraquinone isomers, although there is a marked effect upon their differential affinities toward single-stranded, duplex and triplex DNA forms. The mechanistic consequences of the altered discrimination profiles would be difficult to characterise from biological or cellular studies, particularly using current PCR-based TRAP assay methods where, for example, duplex–drug binding can often demonstrably impair or prevent *Taq* amplification. Further refinements of the telomerase assay protocol will be required to establish the effect(s) of candidate therapeutic agents upon the various DNA constituents implicated in the mechanism of chromosomal telomere extension by telomerase.

Experimental data emerging from our laboratory suggest that the global DNA binding profile of a candidate ligand, usually ignored or neglected in drug design exercises, can be engineered to (i) improve the structural selectivity for tetraplex binding, and hence telomerase inhibition, (ii) reduce or ameliorate unwanted cytotoxicity, and (iii) optimise the therapeutic index as a potential antitumour agent. It is clear that a >10^3-fold improvement in tetraplex affinity will be required to stall telomerase function if the four-stranded DNA is to present an effective blockage for telomeric elongation. However, this must not be achieved at the expense of untoward DNA duplex binding as we have now established a firm linkage between duplex binding and *in vitro* cytotoxicity for the current tetraplex-directed inhibitors. Judicious selection of both the binding chromophore and the complexion of any associated side-chain(s) (e.g., cationic versus anionic substituents, pK_a, hydrophobicity, etc.) should enable these characteristics to be optimised to maximise differential tetraplex/duplex binding. Ideally, it may even be feasible to completely prevent binding to double-stranded DNA. The marked cytotoxicity associated with all reported agents of this class has prevented a demonstration of genuine antitumour activity in whole cells by the proposed telomerase inhibitory mechanism. Significantly, despite reports of activity or 'potent' enzyme inhibition *in vitro* using tumour cell extracts [e.g., refs 57–63,82] there has been a signal failure to achieve telomere shortening in cancer cells. Any tetraplex-directed ligand must be present in cells throughout several doubling times to prevent immortalisation due to the maintenance of telomeric length by telomerase. This requirement for a continuous and sub-toxic (or ideally non-toxic) drug exposure poses a major difficulty for a successful therapeutic realisation of this antitumour strategy.

The powerful techniques now becoming available, particularly equilibrium dialysis and calorimetric experiments that use controlled thermodynamic conditions, can be used to augment molecular modelling in drug development exercises. As outlined, this combined rational strategy will result in improved generations of therapeutically active molecules that can be effectively tailored for the selected nucleic acid biotarget. Thus, it is now possible to realise genuine structural selectivity for tetraplex-form DNA, with the realistic prospect of targeting a key enzyme (telomerase) implicated in the development of most tumours. This is an exciting phase for the development of DNA-directed therapeutic agents, particularly as disease-related gene targets are emerging from the Human Genome Project. The wealth of knowledge accumulated in the development of earlier DNA-interactive molecules can now be used to elicit a biological response with defined gene targets or organised DNA assemblies that play a vital role in cellular progression or transformation.

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ABBREVIATIONS

AQ = Anthraquinone

ITC = Isothermal titration calorimetry

TRAP = Telomeric repeat amplification protocol

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