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Review paper

Telomerase inhibitors: targeting the vulnerable end of cancer?

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In the past decade, a great deal has been learnt about the maintenance of telomeres in mammalian cells by the specialized reverse transcriptase, telomerase, and its associated proteins. The catalytic component of telomerase, hTERT, appears to be selectively activated in the vast majority of tumors relative to most somatic cells suggesting that its inhibition may result in antitumor effects. Although beset with some unusual issues as a drug target, recent 'target validation' studies using hTERT dominant-negative and antisense approaches strongly support the view that potent and selective telomerase inhibitors will induce inhibitory effects on tumors, especially in those possessing relatively short telomeres. Inhibitory strategies have focused on three main areas: antisense molecules (oligonucleotides, RNA molecules, ribozymes and peptide nucleic acids) directed against the hTR RNA component of telomerase, small molecule reverse transcriptase inhibitors (e.g. azidothymidine), and, probably most advanced, small molecules capable of interacting with and stabilizing four-stranded (G-quadruplex) structures formed by telomeres. G-quadruplex interactive agents that inhibit telomerase at sub-micromolar concentrations in cell-free assays have been described. Lead optimization and preclinical whole-cell and animal antitumor and pharmacology studies are now progressing which should result in the first generation of telomerase inhibitors being evaluated in the clinic within the next few years. [© 2000 Lippincott Williams & Wilkins.]

Key words: Telomerase inhibitor.

Introduction

The current paradigm for anticancer drug discovery involves progression from the initial identification of novel cancer-associated genes/proteins that are uniquely and vitally important to cancer cells.[1] Exploitation of these novel targets (e.g. by appropriate inhibition or activation) holds considerable promise in being able to deliver innovative, truly cancer-selective, medicines and thus allow a move away from the poorly selective anti-proliferative molecules that comprise the majority of currently available anticancer drugs. Together with targets within pathways involved in tyrosine kinase-mediated signal transduction, cell cycle control and apoptosis,[1] telomeres and telomerase have also been proposed as an attractive cancer drug target.[2] The purpose of this review is to summarize the key biological properties of telomerase in a cancer therapeutics context, to discuss some of the drug discovery issues raised and to describe various strategies being employed to inhibit this enzyme.

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Telomerase biology and cancer

Telomeres are composed of tandemly repeated DNA sequences at the ends of eukaryotic chromosomes, one strand is rich in guanines (5′-TTAGGG-3′ in humans) and protrudes beyond the complementary cytosine-rich strand.[3] Telomeres, which are approximately 10 and 15 kb in length in blood and sperm, respectively, at birth in humans, protect (cap) the chromosome ends against degradation and fusion with other ends and from being recognized as damaged DNA.[3] However, in the absence of mechanisms for maintaining telomere length, about 100 bases of telomeric DNA is lost at every cell division, due to the 'end-replication' problem.[4,5] This is because conventional DNA polymerases cannot fully replicate the 3′-end of linear molecules during discontinuous lagging strand synthesis. Originally identified in *Tetrahymena*[6,7] and subsequently in human HeLa cells,[8] it is now known that the loss of telomeric DNA may be counterbalanced by the ribonucleoprotein (RNP) enzyme, telomerase, which synthesises the G-rich strand of telomeres by adding single-stranded TTAGGG repeats.

The explosion of interest in telomerase from a cancer context arose from a pivotal study by Kim and colleagues[9] demonstrating a specific association of human telomerase activity with immortal cells and cancer. Using a new PCR-based highly sensitive assay (TRAP—telomeric repeat amplification protocol) it was shown that telomerase activity was absent in normal human somatic tissues (22 normal somatic cell cultures and 50 normal or benign tissues) but reactivated in cancer (98 of 100 cultured immortal cell lines and 90 of 101 tumor biopsies). Subsequently, the observation of telomerase activity in cancer has been confirmed in a variety of tumor types including breast (95% of advanced breast cancers)[10] and gastric.[11] Moreover, high telomerase expression has been correlated with a poor prognosis, in particular in neuroblastoma[12] and node-positive breast cancer.[13]

Human telomerase is now known to consist of at least three core components, an RNA domain (hTR),[14] a catalytic protein domain (hTERT, hTRT, hEST2 and TP2)[15–17] and a further protein (TP1, telomerase-associated protein 1, hTEP1).[18] The RNA subunit of 451 nucleotides provides a template (nucleotides 46–56; CUAACCCUAAC) for the addition of telomeric repeats.[14] The hTERT protein belongs to the reverse transcriptase family of enzymes and shares homology with a 123 kDa protein cloned from the ciliated protozoan *Euplotes aediculatus*, the EST2 protein from *Saccharomyces cerevisiae* and the Trt 1 protein from the fission yeast, *Schizosaccharomyces pombe*.[15–19] TP1 shares homology with the smaller p80 telomerase protein of *Tetrahymena*,[19] a rat homolog (TLP1) has also been identified.[20] Whereas hTR and TP1 are expressed in normal human tissues, the expression of hTERT is the rate-limiting component in being repressed in most normal somatic tissues after birth but becoming activated in telomerase-positive immortal cell lines and tumors.[15,16] Moreover, ectopic expression of hTERT into telomerase-negative cells has been shown to be sufficient to restore telomerase activity, increase telomere length and, notably, extend cellular life-span by at least 20 doublings.[21] Furthermore, hTERT cooperates with the two oncogenes *ras* and SV40 large T antigen to convert normal human cells into transformed, tumor-forming cells.[22] These data all suggest that hTERT is essential for sustained tumor growth and is not merely a secondary marker of the transformed state.

In addition to the telomerase core proteins, various duplex telomere-binding proteins have been identified. These include TRF1, which possesses a Myb-like domain that recognizes a binding site centered on the sequence GGGTTA and has been proposed to be involved as a negative regulator of telomere length.[23,24] Long-term overexpression of TRF1 resulted in progressive telomere shortening while, conversely, expression of a dominant-negative TRF1 mutant caused telomere elongation.[23] Another protein, TRF2 (telomeric repeat binding factor 2), protects human telomeres from end-to-end fusions,[25] possibly through the remodeling of linear telomeric DNA into large duplex loops (t-loops) *in vitro*.[26,27] These t-loops appear to be formed through invasion of the G-strand overhang (which has been shown to be 200 ± 75 nucleotides in normal human chromosomes[28]) and sequestration into the duplex telomeric repeats.[26,27] Notably, inhibition of TRF2 led to a loss of the G-strand overhang and induction of the ATM/p53-dependent DNA damage checkpoint pathway leading to cell cycle arrest and apoptosis.[29] A further protein, termed Tankyrase, is a poly(ADP-ribose) polymerase (PARP) which catalyzes ADP-ribosylation of TRF1, leading to dissociation of TRF1 from telomeres.[30] From *in vitro* studies, the assembly of active hTERT has also been shown to require the molecular chaperones p23 and heat shock protein 90 (Hsp 90).[31] Other proteins recently shown to be associated with the RNP enzyme are Ku proteins (involved in telomere maintenance)[32] and dykserin (involved in assembly of the RNA component),[33] bringing the total mass of the RNP to around a megadalton.

Recent studies indicate that telomerase activity in cells is reversibly regulated, either positively or negatively (for a review, see Liu[34]). The hTERT promoter contains binding sites for several transcription factors; in particular, the proto-oncogene c-Myc has been shown to activate telomerase.[35,36] Telomerase is also activated by the anti-apoptotic protein, BCL-2,[37] and the human papillomavirus (HPV) type-16 E6 protein, probably through interaction with Myc.[38] The level of activity of hTERT is also determined by post-translational phosphorylation/dephosphorylation. Phosphorylation, by serine/threonine kinases protein kinase C (PKC)-α or Akt kinase/protein kinase B (PKB) (on serine 824) increases hTERT activity, whereas dephos-phorylation by protein phosphatase 2A (PP2A) is inhibitory.[39–41] This raises the possibility of using PKC-α or Akt kinase inhibitors alone, or in combination with other telomerase inhibitors, to reduce hTERT activity in tumors (*vide infra*).

Conversely, there are various mechanisms by which hTERT appears to be down-regulated. These include repression activity located on chromosome 3 identified by chromosome transfer into telomerase-positive human breast cancer cells[42] (and also chromosome 7[43]). Overexpression of full-length retinoblastoma (Rb) protein has been shown to down-regulate telomerase activity (which is reduced in S-phase cells).[44] Further-

more, at least in some cells, DNA methylation of a dense CG-rich CpG island (and possibly histone deacetylation) of the *hTERT* gene promoter results in *hTERT* repression.[45,46] It has also been shown that *hTERT* is regulated by alternative splicing.[47]

Telomerase: a good anticancer drug target?

The observations of telomerase activity in tumors paralleled with no activity in normal cells[9] have led many to propose the enzyme as an attractive anticancer drug target.[2,48–53] However, in comparison to most other areas of cancer drug hunting, the development of telomerase inhibitors has been controversial and beset with a variety of special challenges. Issues of concern include: (i) the concept of requiring a significant lag-phase and prolonged inhibition in order to reduce telomeres to critical lengths; (ii) the detection of telomerase activity in some normal cells/tissues (such as proliferative hematopoietic T and B stem cells and the testis[54–56]) raising the issue of possible side-effects; and (iii) the existence of alternative mechanisms (the ALT pathway) to retain telomere length in a small proportion of tumors,[57,58] raising the possibility of drug resistance to inhibitors. However, as described below, there are now compelling data which provide some countenance to each of the above three critical issues and indicate that telomerase inhibitors should be of value in the treatment of cancer, especially when administered chronically, post-surgery or as cytotoxic chemotherapy.

Target validation

In common with drug development approaches involving other new anticancer drug targets, target validation by means of a combination of mouse gene knockouts, antisense and hTERT dominant-negative strategies, have been of particular value. Mouse 'knockout' studies where the RNA component (mTR) has been deleted, revealed that although these mice did not show any major phenotypic changes over the first four to six generations,[59] by the seventh generation defective spermatogenesis (resulting in infertility) and a significant reduction in the proliferation of hematopoietic cells in the bone marrow and spleen was reported.[60] These findings indicate that telomerase/telomeres is involved in the maintenance of genomic integrity and in the long-term viability of high-renewal organs. A possible explanation for the delayed phenotype lies in the observation that mouse telomeres are considerably longer (5–10 times; 20–150 kb) than human telomeres.[61,62]

The original antisense approach used a vector expressing antisense to the hTR RNA component stably transfected into HeLa cancer cells.[14] Fitting with the model of requiring gradual telomere erosion with each cell division, cell crisis in the antisense-treated cells occurred after 20–26 population doublings (with around a 30% reduction in mean telomere length).[14] In another study, using transfection into human glioma cells, two distinct effects, apoptosis or differentiation, were observed after 30 population doublings.[63]

Important recent studies have provided even more compelling validation of the target. To address the importance of hTERT in the growth of tumor cells, a dominant-negative, catalytically inactive, form of hTERT was constructed (by substitution of amino acids aspartic acid 710 and valine 711 with alanine and isoleucine, respectively) and ectopically expressed in various cancer cell lines.[64,65] These pivotal studies showed that the dominant-negative construct completely inhibited telomerase activity, telomeres shortened and, especially in tumor cells possessing short telomeres, caused apoptosis/cell death. The onset of cellular arrest was related to their initial telomere length: in LoVo colon cancer cells (mean telomere length of 2–3 kb) dominant-negative hTERT transfected cells did not proliferate long enough to reach confluence, while in 36M ovarian cells (mean telomere length of 5–7 kb) the transfected cells showed no change in growth rate until arrest was seen at 30–40 days.[64] Apoptosis occurred by a p53-independent pathway and, moreover, tumorigenicity in nude mice was eliminated. In a cell line (GM847) which maintains its telomeres by the ALT pathway, no such effects were observed.[64] These data, emphasizing the importance of telomere length in determining outcome, may provide an explanation for some previous reports in Burkitt's lymphoma and malignant glioma cells subjected to antisense telomerase inhibition where, rather than the predicted lag in response, rapid apoptosis was observed.[66,67] This essential interplay between telomerase activity, maintenance of telomere length and apoptosis has also recently been highlighted in human cells where resistance to apoptosis was conferred in telomerase-positive cells by experimentally elongating their telomeres.[68]

In summary, these data suggest that potent and selective telomerase inhibitors might confer significant antitumor effects, especially in tumors possessing relatively short telomeres. This highlights the necessity for the development of rapid, highly sensitive and accurate methods for determining telomere lengths in tumors. The most widely used technique is Southern

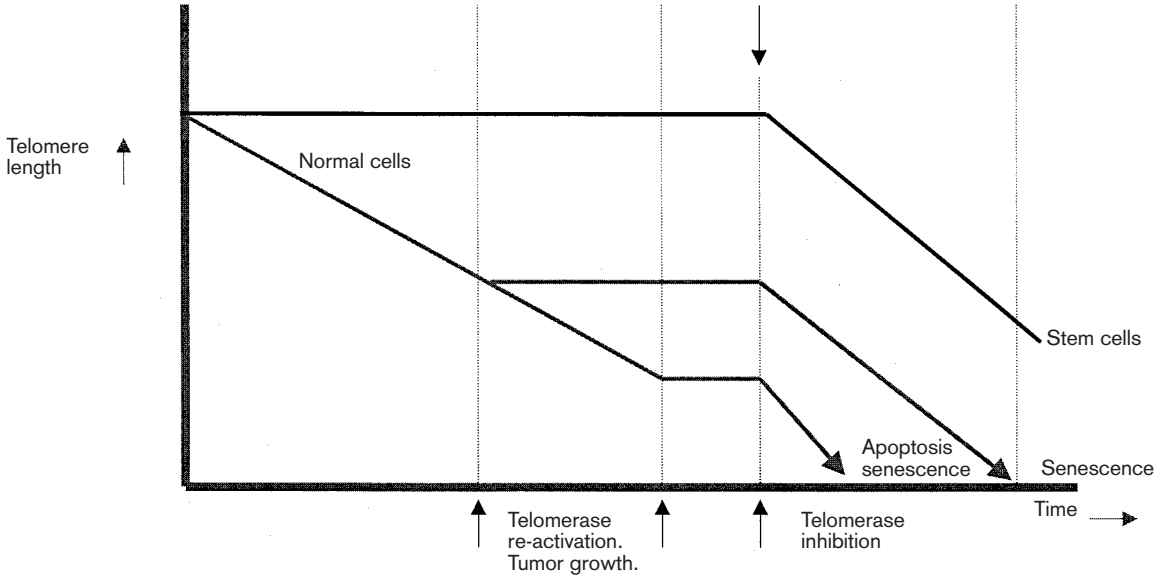

Figure 1. Hypothetical clinical strategy for optimal use of telomerase inhibitors.

blotting to detect the terminal restriction fragment (TRF) although alternative methods such as slot blotting or Flow-FISH may prove more useful in this setting.[69,70] There is now evidence from cell lines (including across the National Cancer Institute 60 line panel[71]) that mean telomere lengths do vary across cell lines and are generally considerably shorter than those of normal somatic cells.[5,72] Encouragingly, if such tumors could be identified, then the application of inhibitors to these selected patients should result in relatively rapid senescence or apoptosis in tumors well before any toxicities resulting from telomere erosion in normal organs with longer telomeres occurs (Figure 1). A recent report measured telomere length in patients presenting with lymphomas, including consecutively in the same patient at presentation and relapse; a subset of patients with tumors possessing shortened telomeres at relapse was apparent.[73] Finally, to date, there has been no evidence of drug-resistant revertants emerging (e.g. by the ALT pathway) in cancer cells of epithelial origin exposed to telomerase inhibitory strategies.

Strategies for the inhibition of telomerase/telomeres

Armed with some understanding of the genes/proteins involved in maintaining telomeres in human cancer cells, a variety of approaches to telomerase inhibition have been proposed (Figure 2). These include various antisense approaches to hTR, hTERT and accessory proteins, reverse transcriptase inhibitors, and small molecules targeted at the telomeric G-rich overhang.

Antisense approaches

In addition to the target validation antisense studies described above (using stable transfection of antisense constructs into tumor cells), numerous other antisense strategies have been employed. Typically, this has involved targeting of the telomerase 11-base RNA template with either phosphorothioate DNA,[66] oligonucleotides linked to a 2′,5′-oligoadenylate (2–5A),[67] RNA oligonucleotides (2′-*O*-methyl RNA),[74] catalytic RNA hammerhead ribozymes[75,76] and peptide nucleic acids (PNAs).[77–81] Telomerase activity has also been depleted in leukemia cell lines using antisense oligonucleotides directed against c-*myc* RNA.[82] These studies have provided a useful mechanistic insight into the cellular effects of inhibition of the telomerase RNA subunit and provide further target validation support to the above-described knockout and dominant-negative studies. However, problems of cell uptake (cationic lipids or electroporation are often required to achieve intracellular delivery) and stability (oligonucleotides are highly susceptible to protease or nuclease degradation) in

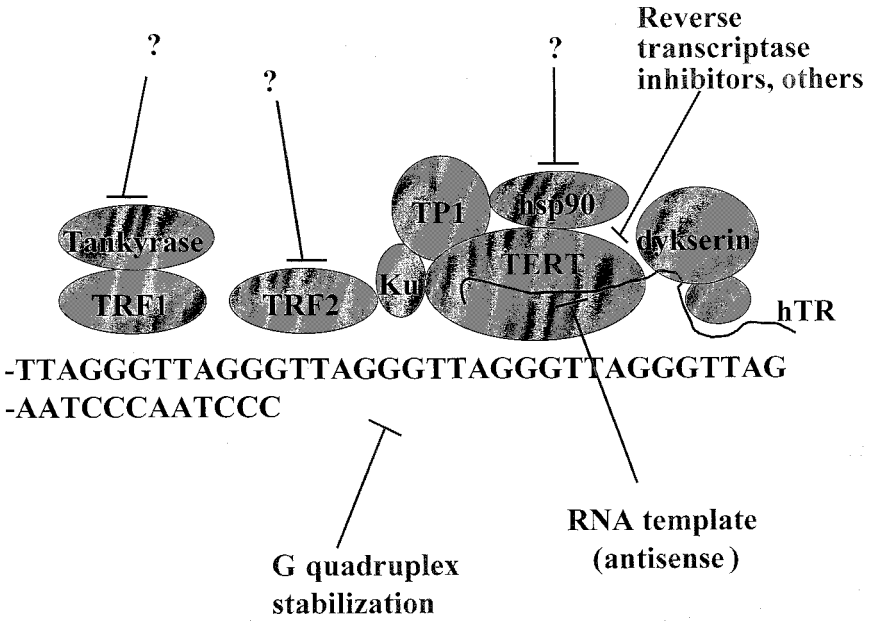

Figure 2. Telemere/telemerase components and possible points of inhibition.

comparison to 'small molecule' inhibitors (see below) may limit the clinical application of these antisense approaches. Regardless, some of these preclinical studies have shown some promising effects, including two where *in vivo* antitumor effects in mice have been reported.[66,67] Some reduction in tumor size relative to controls was observed at 10 days in animals bearing a Burkitt's lymphoma using a continuous infusion (50 μg/mouse/day) of a hexameric phosphorothioate oligonucleotide.[66] In a human malignant glioma s.c. xenograft model, direct intratumoral injection every day for 7–14 days of a 19-mer 2–5A linked oligonucleotide (which activates the endoribonuclease, RNase L) led to a significant reduction in tumor growth at 7 days.[67] However, direct effects on telomerase or telomeres in the xenografts were not proven in either of these studies. Experiments using PNAs scanning the 11-base RNA template have shown particularly potent inhibition of telomerase (with IC_{50} in the low nanomolar range).[78] More recently, improved methods of delivering PNAs into cells using cationic-lipids and PNA–DNA hybrids has been reported with DU145 prostate cancer cells.[80] This has culminated in an important antisense study using both PNAs and 2′-*O*-methyl RNA where exposure to cells led to telomerase inhibition, progressive telomere shortening and onset of apoptosis, dependent upon initial telomere length.[81] Interestingly, once the inhibitors were removed, telomeres rapidly regained their lengths suggesting that (i) telomerase inhibition must be maintained until the 'critical' telomere length is reached and (ii) the telomeres of stem cells that are affected during therapy may rapidly recover once therapy is completed.

Small molecule inhibitors

Reverse transcriptase inhibitors. The realization that hTERT belongs to the reverse transcriptase family of enzymes has prompted the use of known reverse transcriptase inhibitors, particularly 3′-azido-2′,3′- dideoxythymidine (AZT), as telomerase inhibitors, initially in *Tetrahymena*.[83] Subsequently, several cell-free (TRAP assay) and whole-cell-based studies have shown AZT to inhibit telomerase,[84–88] in some cases with telomere shortening in cells.[84,87] Other nucleoside analogs, such as carbovir[85] and especially dideoxyguanosine (ddG),[84,88] are also potent inhibitors. Additional studies have shown that 7-deaza nucleotides (7-deaza-dGTP and 7-deaza-dATP) are potent cell-free telomerase inhibitors (IC_{50} values of around 10 μM).[89] However, selectivity for telomerase versus other polymerases is a key issue and limitation with this approach (including the fact that 'false-positive' telomerase inhibitors may be identified from the widely used TRAP assay through compound inhibition of Taq polymerase).

G-quadruplex interactive agents. It was shown some time ago that the guanine (G)-rich telomeric overhangs *in vitro* may fold into four-strand G-quadruplex (or tetraplex) structures and may then

directly inhibit telomere elongation by telomerase.[90] Therefore, small molecules that preferentially interact with and stabilize G-quadruplexes have been proposed as telomerase inhibitors.[91,92] An initial collaboration between Stephen Neidle and colleagues within our Institute and Laurence Hurley and co-workers (Austin, TX) identified the first small molecule inhibitors of human telomerase, anthraquinones, based on G-quadruplex interaction.[93] The most potent inhibitor (#1, Figure 3) inhibited telomerase in cell free assays with an IC_{50} of 23 μM.

Subsequently, both groups have actively pursued the development of more potent G-quadruplex interactive compounds resulting in the discovery of other regioisomers of anthraquinones,[94,95] amidofluorenones,[96] acridines,[97] a mono-substituted tetracyclic compound[98] and cationic porphyrins[99] (Figure 3). The most potent compounds inhibit telomerase in the cell-free TRAP assay in the low micromolar region. A particular attribute of telomerase inhibitors (in contrast to classical anticancer cytotoxics) is that they should not induce short-term acute cytotoxicity but rather should induce delayed cell senescence or apoptosis, the length of the delay being dependent upon initial telomere length (*vide supra*). Therefore, we have employed a strategy where a direct comparison of telomerase inhibitory potency in the TRAP assay (excluding those compounds that non-specifically inhibit Taq polymerase) and acute cytotoxicity (using a 4-day sulforhodamine B growth inhibition assay and three human ovarian carcinoma cell lines) is made.[49,94–98] Compounds are sought (and selected for further whole cell studies and *in vivo* antitumor and pharmacology studies) which exhibit a wide differential between potent telomerase inhibitory activity and possess low acute cytotoxicity (believed to be indicative of effects on duplex DNA). Such whole-cell and *in vivo* studies are now ongoing with lead inhibitors; cellular senescence has been reported using

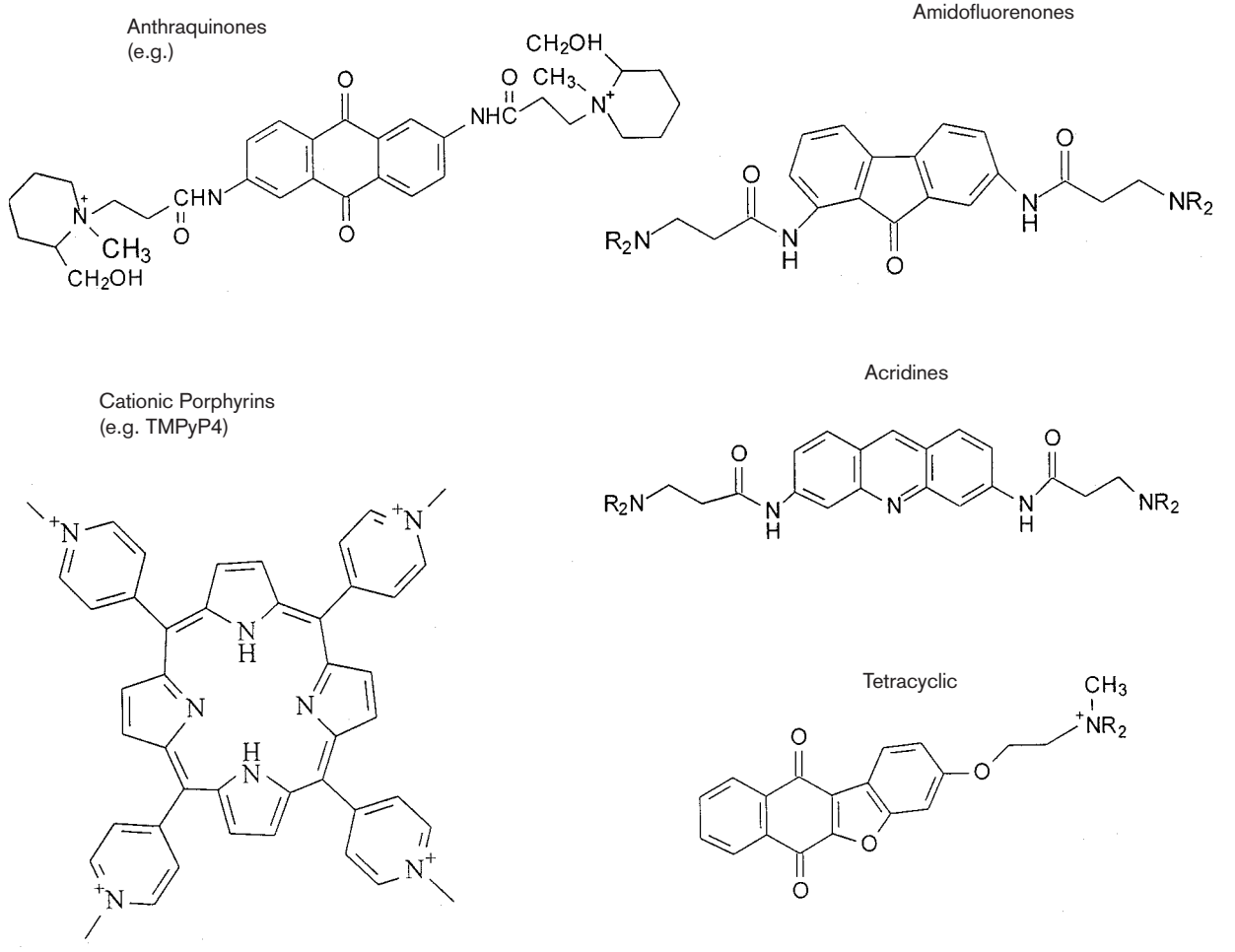

Figure 3. G-quadruplex inhibitors.

non-acute cytotoxic concentrations of acridine-based G-quadruplex inhibitors after 14 days exposure of 21NT human breast cancer cells.[51]

Whole cell studies have been reported for the lead porphyrin-based inhibitor, TMPyP4.[100–102] Using sub-acute cytotoxic concentrations, growth arrest of MCF-7 human breast cancer cells was observed over 15 days (only 10 population doubling times).[100] While a reduction in telomerase activity was observed, this was not accompanied by any observable reduction in telomere length, however.[101] Furthermore, effects against normal cells have also been shown to occur with this compound.[102]

Although there is no absolute evidence to confirm the existence of G-quadruplex DNA *in vivo*, there is evidence that these molecules do indeed bind to quadruplexes. In addition, the leads from this class of telomerase inhibitors are probably closest to the clinical interface. While no crystal structure is yet available for a G-quadruplex ligand complex, a solution NMR-based model has been proposed for the quadruplex inhibitor PIPER (3,4,9,10-perylenetetracarboxylic acid diimide).[103] Also, consistent with the need for the four repeats of the d(TTAGGG) telomeric hexanucleotide to be formed prior to G-quadruplex formation, inhibition with inhibitors only occurred after three to four telomere repeats had been synthesized.[93] The more rapid identification of additional quadruplex selective versus duplex DNA binders may be assisted by a recently described competition dialysis-based assay where the previously described compound DODC (3,3′-diethyloxadicarbocyanine) was shown to preferentially bind triplex rather than tetraplex oligonucleotides.[104]

Miscellaneous small molecules. In recent years, a number of additional small molecule inhibitors of telomerase have been identified (Figure 4), some by means of high-throughput TRAP assay-based screening.[105] In most cases, the mechanism of telomerase inhibition is unclear although it is proposed that the isothiazolone derivatives (the most potent of which inhibit telomerase with IC_{50} values of 1 μM in the TRAP assay) may interact with critical cysteine residues near the hTERT active site.[106] Other inhibitors include FJ5002, a rhodocyanine (telomerase IC_{50} of 2 μM) which also induced telomere erosion and senescence in a human leukemia cell line after prolonged exposure.[107] Another inhibitor (EGCG, Figure 4) is a catechin component of green tea (telomerase IC_{50} of approximately 1 μM) and induced

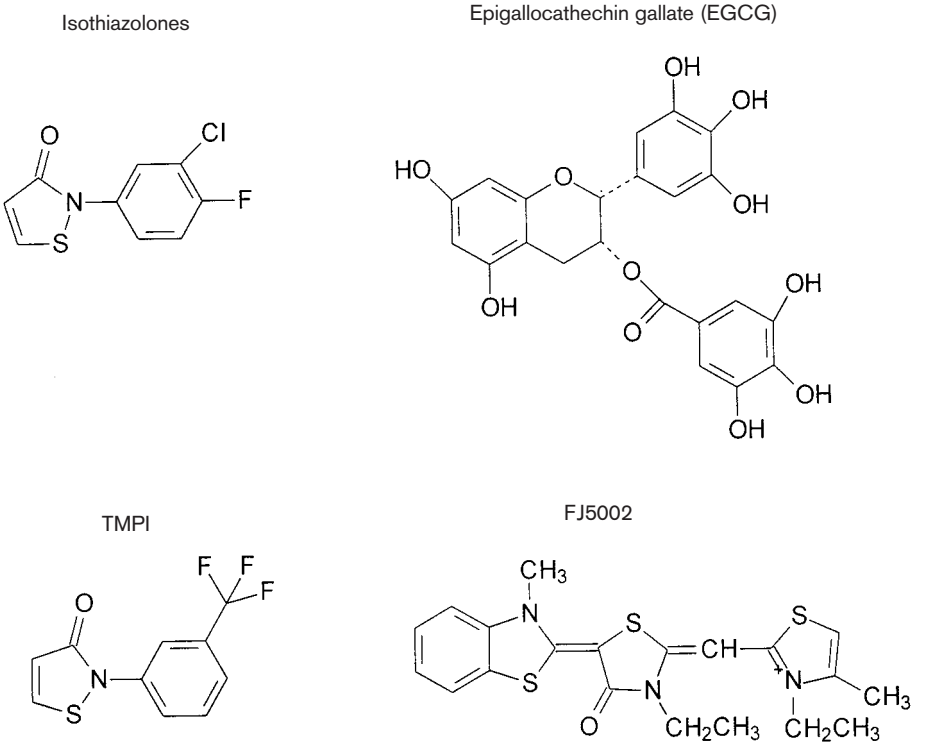

Figure 4. Additional inhibitors of telomerase.

senescence in two human cancer cell lines.[108] The PKC inhibitors bisindolylmaleimide I and H-7 may exert their anti-telomerase effect through affecting the phosphorylation status of hTERT (*vide supra*).[109]

Other experiments have shown that commonly used cancer chemotherapy cytotoxics, especially cisplatin, cause a reduction in intracellular telomerase following exposure.[110,111] However, in a pharmacology context, relatively high concentrations of cisplatin are required and it does not directly inhibit telomerase in the cell-free TRAP assay,[110] thereby making it difficult to discriminate between a direct effect on the telomerase/telomere machinery and a more generalized shut-down as part of cell death. The quinolone antibiotics ofloxacin and levofloxacin also reduced telomerase activity in cells at relatively high concentrations while not inhibiting directly in the TRAP assay.[112] Indeed other groups have advocated the loss of telomerase activity as an indicator of tumor response to cytotoxic chemotherapy.[113]

Conclusions

The realization that telomerase represents a cancer-associated protein has stimulated intense interest in terms of both diagnostics as a possible means of early detection of cancer and, moreover, in a therapeutic setting. Pivotal 'proof of principle' target validation preclinical studies utilizing antisense technologies and dominant-negative constructs has recently provided evidence that targeting the telomere/telomerase cellular machinery may confer a significant antitumor benefit. Both cellular senescence and apoptosis have been observed following telomerase inhibition; the effect appearing to be dependent upon initial telomere length (thus indicating that inhibitory strategies should be focused on tumors possessing relatively short telomeres). It is probable that telomerase inhibitors might be best utilized in the clinic as chronic therapy, post-surgery or post-cytotoxic chemotherapy, or even in a chemopreventative setting.

Various inhibitory strategies have been advocated including antisense oligonucleotides and PNA's directed against the telomerase RNA component, small molecule reverse transcriptase inhibitors and, probably most advanced, small molecule inhibitors directed at the telomere (G-quadruplex interactive agents). Rational design of inhibitors directed at the active site, catalytic domain of hTERT might be feasible when the high-resolution crystal structure is solved.

The telomerase inhibitor field is now entering a particularly exciting phase as the first generation of inhibitors enter lead optimization and phase I clinical evaluation. Until such time that 'proof of principle' clinical trials are conducted with highly potent and selective inhibitors and with appropriate pharmacodynamic monitoring (i.e. measurements of hTERT expression and telomere length reduction), the hot debate will continue as to whether telomerase inhibitors will be of value as anticancer drugs or not.

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