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# Telomeres and telomerase as targets for anticancer drug development

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## Contents



**Abstract**

In most human cancers, the telomere erosion problem has been bypassed through the activation of a telomere maintenance system (usually activation of telomerase). Therefore, telomere and telomerase are attractive targets for anti-cancer therapeutic interventions. Here, we review

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a large panel of strategies that have been explored to date, from small inhibitors of the catalytic sub-unit of telomerase to anti-telomerase immunotherapy and gene therapy. The many positive results that are reported from anti-telomere/telomerase assays suggest a prudent optimism for a possible clinical application in a close future. However, we discuss some of the main limits for these approaches of antitumour drug development and why significant work remains before a clinically useful drug can be proposed to patients.


*Keywords:* Targeted anticancer therapy; Telomeres; Telomerase

## 1. Introduction

The current trend in research on anticancer drugs is to exploit particular traits or hallmarks unique to cancer cells. Despite the fact that cancer displays a great heterogeneity in clinical behaviour, most human tumours, share a limited set of acquired capabilities that define the malignant state [1]. These include self-sufficiency in growth signal, insensitivity to antigrowth signals, avoidance of programmed cell death, unlimited replicative potential, sustained angiogenesis, tissue invasion and metastasis. Among these hallmarks, the acquisition of unlimited replicative potential is a key step to ensure expansive tumour growth.

Activation of a telomere maintenance mechanism seems indispensable for immortalisation of human cells. Telomeres and telomerase, the protein that allows their maintenance, have therefore been proposed as preferential targets for anticancer drug development. This review highlights recent advances in our understanding of mammalian telomere biology and how it relates to cancer, and discusses current approaches that exploit this knowledge to develop novel antineoplastic drugs.

## 2. Telomere biology and cancer

### *2.1. Unlimited replication potential: the link to telomeres and telomerase*

Normal cultured cells have a finite replicative potential [2] meaning that after a certain number of divisions they stop growing and enter senescence, a stage named mortality 1 (M1). In human fibroblasts the senescence can be bypassed by inactivation of the tumour suppressor genes p53 and pRb. These transformed cells progress through 20–30 doublings before they enter a second state called crisis or mortality 2 (M2). Cells that escape crisis have acquired the ability to divide without a limit, a trait called immortalisation [3]. Over the past decade, emerging evidence has shown that the ends of chromosomes, or telomeres, are essential regulators of life span. Human telomeres which are composed of several thousand repeats of a T2AG3 hexanucleotide sequence element, progressively shorten as normal cells proliferate [4,5], whereas immortalised cells, including most types of tumour cells, maintain a stable telomere length [6]. Thus, telomeres have been proposed to serve as a molecular device that counts the number of cellular divisions and limits life span [7]. In most malignant cells (85–90%), the maintenance of telomeres is achieved by upregulating the expression of the telomerase enzyme, which adds hexanucleotide repeats onto the ends of telomeres [8] whereas, 10–15% of the remainder tumours or tumour cell lines maintain the length of telomeres through a telomerase-independent alternative lengthening of telomere (ALT) mechanism [9,10], a process implicating homologous recombination [11]. However, the maintenance of telomere length above a critical threshold through either mechanism permits unlimited replication of cells. The ALT phenotype is characterized by heterogeneous telomere lengths and the presence of a variant form of the promyelocytic leukaemia (PML) nuclear bodies at the telomeric level, also called APBs (ALT-associated PML bodies) [12]. Immunofluorescent techniques have demonstrated that APBs bodies, in addition to the PML protein, contain telomeric DNA with their usual telomere-associated proteins (e.g. TRF1, TRF2), but also proteins that are implicated in double strand break repair and homologous recombination such as RPA, Pre11/Nbs1/Rad51 and Rad52 [12]. Further, it has been proposed that tumours presenting ALT-phenotypes have potentially a higher chromosomal instability than telomerase positive tumours [13–15]. However, the clinical evolution and sensitivity to treatment of ALT-tumours are still poorly described despite the reported significance of the ALT-pathway in, for instance, sarcomas [16].

### *2.2. Telomeres, a multi-protein complex*

The extremities of eukaryotic chromosomes are composed of specialized DNA nucleoprotein complexes termed telomeres (Fig. 1). Human telomeres consist of a variable number of tandem repeats of the T2AG3 sequence together with a group of specific proteins, and are therefore of variable length. At the 3′ end, the G rich strand of the telomere forms a single stranded extension. Recent ultrastructural evidence in vitro suggests that the telomere repeated sequence folds back on itself to form a duplex loop structure termed T-loop [17]. Both telomeric DNA and telomere-associated proteins have an essential role in stabilizing chromosome ends by forming a cap structure that protects chromosome ends from exonucleolytic degradation and terminal fusions. Some telomere-associated proteins bind directly to the T2AG3 DNA repeats, whereas others are associated with the telomere via protein–protein interactions (Fig. 2). In humans, the

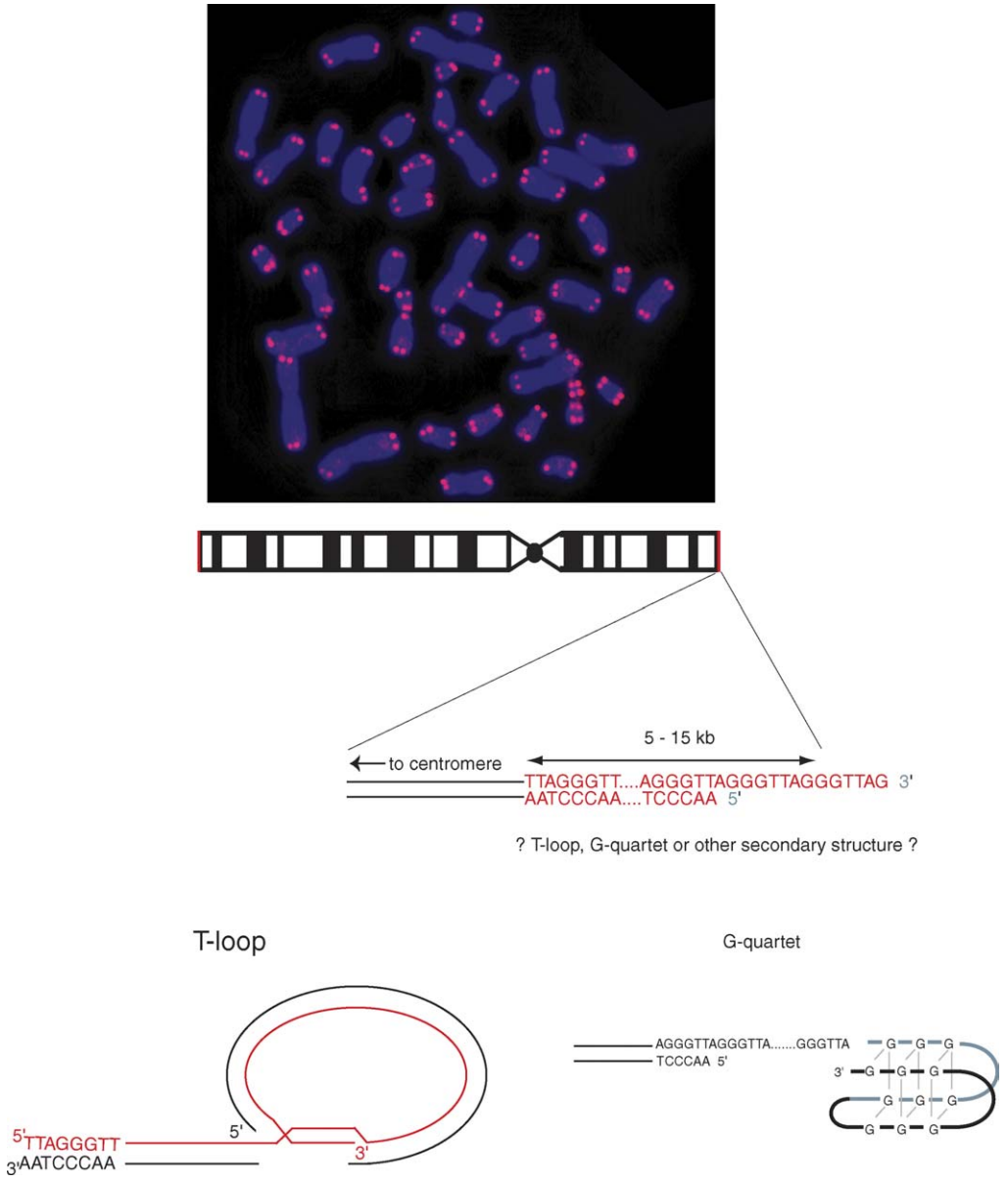


Fig. 1. Telomere sequence and structure in humans. The telomeres are visualized by fluorescent in situ hybridisation (upper picture) using a telomere-specific PNA probe (in red) on metaphase chromosome spreads. The chromosomes are counter-coloured by DAPI (in blue). The telomeres consist of a variable number of tandem repeats of the T2AG3 sequence and are therefore of variable length. At the 3′ end, the G rich strand of the telomere forms a single stranded extension. The telomere repeated sequence folds back on itself in vitro to form a duplex loop structure termed T-loop, but structures such as G-quartets at telomere 3′ ends are possible.

TRF1 and TRF2 proteins specifically bind double stranded telomere sequences through a Myb-like helix/turn/helix motif [18–20] whereas the POT1 protein associates with the 3′ single stranded overhang through its oligonucleotide binding (OB) fold motif [21,22].

Both TRF1 and TRF2 are involved in regulating telomere length in vivo (Table 1). Overexpression of TRF1 or TRF2 leads to a gradual shortening of telomeres in telomerase positive human cells [23–25]. However, overexpression of TRF2 but not TRF1 in telomerase negative human cells resulted in accelerated telomere shortening [26] suggesting that these proteins act through distinct pathways. Accordingly, the expression of a TRF1 or TRF2 dominant negative mutant has shown different effects on telomeres in human cells. When a dominant negative mutant of TRF1, which prevents the association of endogenous TRF1 with telomeres, was overexpressed the telomere gradually elongated suggesting that the primary function of TRF1 is to negatively regulate telomere length [25]. Furthermore, different levels of TRF1 did not affect the rate of telomere shortening in telomerase negative cells, indicating that TRF1 alters telomere length by affecting the telomere elongation step [26]. It has been suggested that TRF1 controls negatively telomerase activity in cis [23]. This was demonstrated in an experiment that

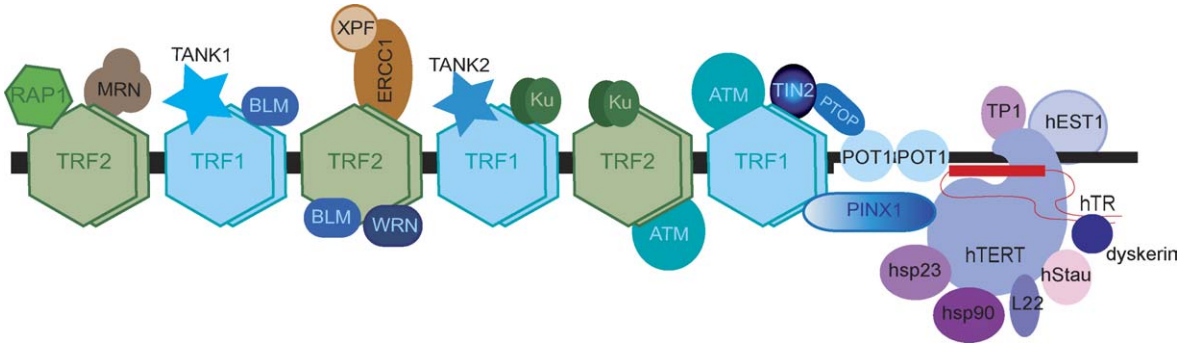


Fig. 2. Telomere-associated proteins in humans. Both telomeric DNA and telomere-associated proteins have an essential role in stabilizing chromosome ends by forming a cap structure that protects chromosome ends from exonucleolytic degradation and terminal fusions. Some telomere-associated proteins bind directly to the T2AG3 DNA repeats, whereas others are associated with the telomere via protein–protein interactions. In humans, the TRF1 and TRF2 proteins bind double stranded telomere sequences through a Myb-like helix/turn/helix motif whereas the POT1 protein associates with the 3′ single stranded overhang. TRF1, TRF2 and POT1 interact together and associate directly or indirectly with other proteins. TRF1 interacts with the poly(ADP-ribose) polymerase Tankyrase 1 and 2. TRF1 also directly binds TIN2, and interacts with POT1 through a TIN2/PTOP interaction. Another direct TRF1 interacting factor is PINX1, a protein that binds to hTERT. TRF1 has also been shown to bind a number of proteins involved in DNA repair or checkpoint including, Ku, BLM helicase, and ATM kinase. The TRF2 protein makes a complex with the hRap1 protein. The TRF2–hRap1 complex interacts with the Mre11/Rad50/Nbs1 DNA repair complex, the nucleotide base excision repair endonuclease ERCC1/XPF, the WRN and BLM helicases, Ku heterodimer, and the ATM kinase. Telomerase is composed of the catalytic subunit hTERT and an RNA component (hTR). The subunits associates to form a complex tetramer composed of two RNA subunits and two catalytic subunits. In addition to these core components other proteins that are dispensable for catalytic activity associate with telomerase, including TP1, hsp23, hsp90, hStau, L22 and dyskerin. Finally, the hEst1p protein presumably recruits and activates telomerase at the 3′ end of telomeres.

tethered a LacI-TRF1 fusion plasmid to subtelomeric LacO repeats and that limited the telomere elongation. In addition, it was shown by ChIP experiments on several cell lines that the amount of TRF1 at telomere is proportional to the T2AG3 length [27]. Based on these data and in accordance with the 'counting mechanism' proposed for the regulation of telomere length in yeast [28] the proposed model is that the amount of TRF1 controls in cis the action of telomerase at each telomere [25].

Whereas TRF1 seems directly involved in telomerase regulation, TRF2 is thought to mostly protect chromosome ends. Firstly, TRF2 was proposed to promote the formation of

Table 1
Telomerase-/telomere-associated proteins and their interactions

| Factors | Name in human | Functions at telomere | Interactions with |
|---|---|---|---|
| Telomere specific proteins | | | |
| Telomerase catalytic core | hTR | RNA subunit | |
| | hTERT | Reverse transcriptase subunit | |
| Telomerase accessory factors | EST1A, EST1B | | Telomerase |
| G-tail binding factors | POT1 | Binds T2AG3 using OB-fold | TRF1, TRF2, PTOP, TIN2, Tankyrase 1 |
| Duplex T2AG3 binding factors | TRF1 | Binds telomeres, negative length regulator | POT1, TRF2, TIN2, PINX1, TANK1/2, Ku, BLM, ATM |
| | TRF2 | Binds telomeres, negative length regulator, role in T-loop, chromosome stability | POT1, TRF1, hRAP1, PARP2, TIN2, MRN, ERCC1/XPF, WRN, BLM, Ku, ATM |
| Proteins indirectly binding telomere | hRAP1 | Length regulator | TRF2, MRN |
| | TANK1/2 | PARP activity, TRF1 ribosylation, positive length regulator | TRF1 |
| | TIN2 | Positive length regulator | TRF1, TRF2 |
| | PINX1 | Telomerase inhibitor | TRF1, TIN2 |
| Others | | | |
| DNA repair proteins | Ku70/Ku86 | NHEJ, telomere localisation, negative length regulator, telomere capping | Telomerase, TRF1, TRF2 |
| | DNAPKcs | NHEJ, telomere localisation, telomere capping | |
| | Mre11/Rad50/Nbs1 | Recombination, NHEJ | TRF2 |
| | ERCC1/XPF | NER | TRF2 |
| Helicases | WRN and BLM | Recombination, NHEJ | TRF1, TRF2 |
| Checkpoint proteins | ATM | DNA damage signaling | TRF1, TRF2 |

T-loops [17,29]. Secondly, inhibition of TRF2 by the expression of a dominant negative allele or by RNAi resulted in loss of the 3′ overhang without detectable loss of double stranded telomeric sequences, and end to end fusions generated by DNA ligase IV-dependent non homologous end joining [30–32]. These deprotected telomeres are in turn recognized like double strand breaks and induce a p53 and ATM apoptotic pathway [33].

The role of POT1 in regulating telomere length is less clear. Recently the resolution of the crystal structure of the N-terminal half of POT1 bound to a telomeric ssDNA decamer showed that the telomeric 3′-terminal guanine base is buried in the protein [34], sustaining that POT1 makes the telomere 3′ extremity inaccessible to telomerase and thus probably inhibits telomere elongation as previously predicted [27].

TRF1, TRF2 and POT1 interact together and associate directly or indirectly with other proteins that can also affect the length of telomeres. TRF1 recruits a number of proteins to the telomere (reviewed in [35]). TRF1 interacts and is modified by the poly(ADP-ribose) polymerase Tankyrase 1 and 2 [36–41]. The ADP-ribosylation of TRF1 by tankyrases diminishes its ability to bind telomeric DNA [41]. TRF1 also directly binds TIN2 (TRF1 interacting protein 2), an association that appears to protect TRF1 from tankyrase in vivo and has been proposed to regulate the access of telomerase to the telomeres [42,43]. The TRF1 complex interacts with POT1 through a TIN2/PTOP interaction (PTOP is also called PIP1 or TINT1) [44,45]. A third direct TRF1 interacting factor is PINX1, a protein that binds to hTERT and directly inhibits telomerase activity in vitro [46]. Recently, results coming from yeast studies suggested that PinX1 regulates telomerase by sequestering its protein catalytic subunit in an inactive complex lacking telomerase RNA [47]. TRF1 has also been shown to bind a number of proteins involved in DNA repair or checkpoint control including, the non-homologous end joining protein Ku [48], the BLM helicase [49,50], and the ATM kinase [51,52].

TRF2 has also a number of interacting factors. The TRF2 protein makes a complex with the hRap1 protein [53]. The TRF2–hRap1 complex interacts with the Mre11/Rad50/Nbs1 DNA repair complex [54], the nucleotide base excision repair endonuclease ERCC1/XPF [55], the WRN and BLM helicases [50,56,57], Ku heterodimer [58], and the ATM kinase [51]. Moreover, like TRF1, the activity of TRF2 at telomeres is modulated through an interaction with PARP2 [59].

Finally, the TRF1 and TRF2 complexes seems connected through a simultaneous binding of TIN2, a connection that stabilizes their levels and localization at telomeres and modulates their capping function [43,60].

### 2.3. Telomerase

The terminal replication of chromosomes requires a specialised polymerase, termed telomerase (Fig. 3) since conventional DNA polymerases, responsible for the majority of DNA replication in eukaryotic cells, are unable to synthesise the last stretch of DNA on the lagging strand [61] – a phenomenon known as the ‘end replication problem’. In the absence of telomerase the extreme end of the chromosome is not replicated and the telomeres progressively shorten with every cell division [5]. Telomerase is a unique reverse transcriptase [62,63] composed of both protein subunits (among which the catalytic subunit hTERT in humans [64,65]), and an RNA component (hTR in humans) [66] that serves as the template for the addition of the repeated sequence to 3′ chromosome ends [63,67]. hTR is a member of small nucleolar RNA molecules termed box H/ACA RNAs [68]. The role of the major protein subunit, hTERT, is to catalyse the polymerisation of nucleotides. The subunits associates to form a complex tetramer composed of two RNA subunits and two catalytic subunits [69–71] and are sufficient for catalytic activity both in vitro and in vivo [72,73]. In addition to these core components other proteins that are dispensable for catalytic activity associate with telomerase, including TP1, hsp23 and hsp90 [74–77], hStau, L22 [78] and dyskerin [79]. However, the role of these telomerase associated factors remains to be investigated. More recently, homologs of the yeast Est1p protein, that recruits and activates telomerase at the 3′ end of telomeres, have been identified in human. EST1A and EST1B associate with telomeres and bind telomerase in vitro and overproduction of EST1A affects telomere length and capping [80,81].

Human telomerase is regulated during development and differentiation, mostly through transcriptional control of hTERT. It has been shown that hTERT undergoes alternative splicing [82], and that one deletion splice variant, hTERTalpha, is a dominant negative inhibitor of telomerase activity [83]. Although hTR is ubiquitously expressed in mammalian cells [66], the expression of hTERT is restricted to cells that exhibit telomerase activity indicating that hTERT is the rate-limiting component of the telomerase enzyme [64]. Altogether, these findings suggest that the expression of hTERT is a critical step during malignant transformation. Indeed, three tumour suppressor pathways have been identified as negative regulators of hTERT transcription: Mad1 a repressor of c-Myc; TGFB, acting through SIP1; Menin, binding directly to the hTERT promoter [84]. Other negative regulators have been described: pRB, chromosome 3 transfer, and Wilm’s tumour 1 suppressor gene. The expression of hTERT is also positively regulated notably by MYC, BCL2, E6 human papillomavirus type 16 protein, phosphorylation by PKCa or AKT/PKB (reviewed in [85]).

### 2.4. Telomerase and cancer

The demonstration that telomerase is actually one of the key enzymes for human cells to acquire immortality has come by complementary approaches. First, the ectopic expression of telomerase in telomerase-null, mortal human cells stabilizes telomeres and facilitates immortalisation, a trait already recognized as a crucial step during cell transformation

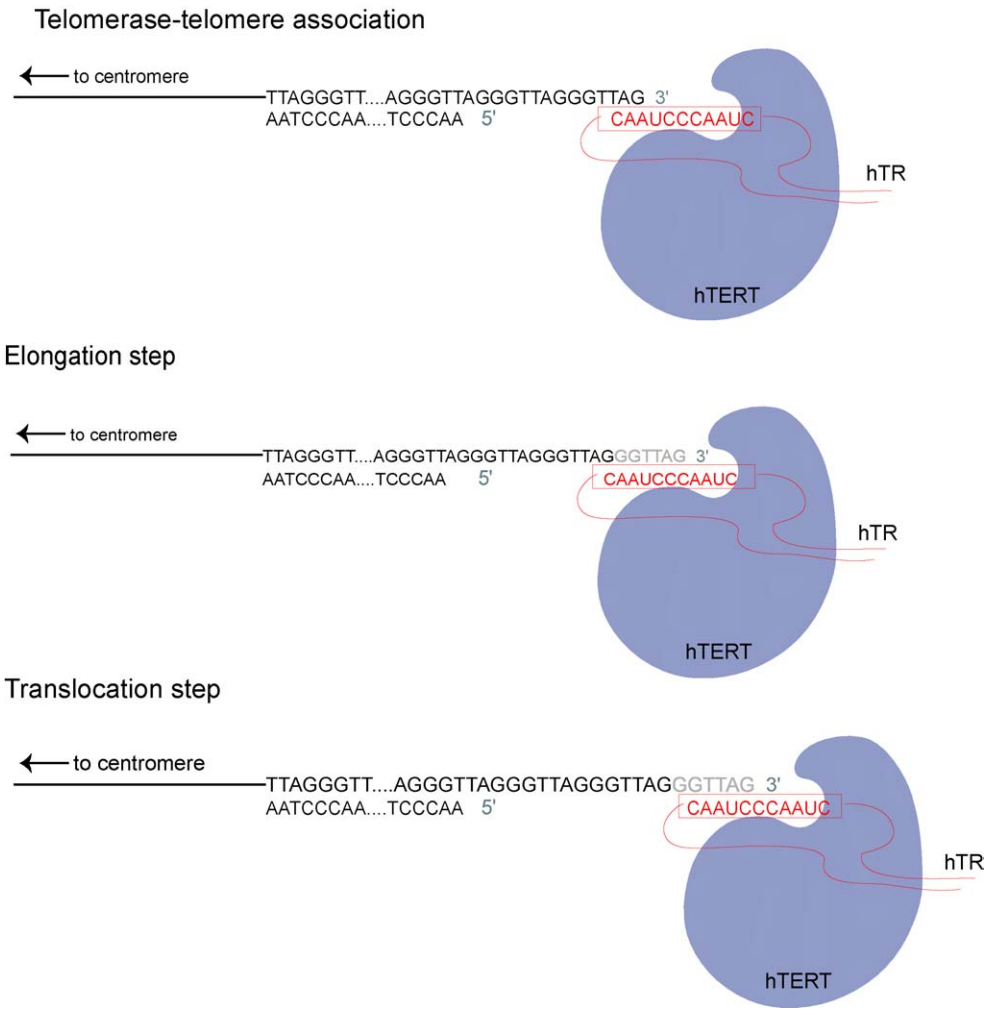


Fig. 3. Human telomerase in action. The terminal replication of chromosomes requires telomerase since conventional DNA polymerases are unable to synthesise the last stretch of DNA on the lagging strand. Telomerase is a reverse transcriptase composed of a catalytic subunit called hTERT and an RNA component (hTR) that serves as the template for addition of nucleotides to the repeated sequence. The role of the protein subunit hTERT is to catalyse the polymerisation of nucleotides (elongation step). Then the telomerase slips one repeat unit towards the 3′ end (translocation step) to start a new elongation step, and so on.

[86–90]. Secondly, the conversion of human fibroblasts or epithelial cells to transformed cancer cells is facilitated by hTERT expression in conjonction with oncogenes (SV40 small T and large T and RAS [91]. Lastly, the inhibition of telomerase in immortal human cancer cell lines leads to apoptosis or senescence [92–95]. Additional clues that telomere-associated events are indeed relevant to carcinogenesis come from the analyses of mice deficient for telomerase showing that in certain genetic contexts, impaired telomere functions can facilitate cancer. For example, the tumour incidence of p16INK4a null mice was reduced in the absence of telomerase [96]. Further, in p53 and telomerase double deficient mice the tumour onset is markedly accelerated [97,98], and the constitutive expression of mTert in Lck-TERT mice leads to increased promotion of lymphoma independently of telomere length maintenance [99]. Higher incidence of both induced and spontaneous epidermal tumors has also been observed in transgenic mice overexpressing mTERT in basal keratinocytes [100].

Therefore, the evidence that most cancer cells activate telomerase whereas normal cells are usually devoid of telomerase activity (with the exception of ongoing proliferating cells such as lymphocytes, basal keratinocytes, intestinal crypt cells, CD34 expressing peripheral blood stem cells, and germline cells) has naturally lead to extensive investigations to detect this protein and its activity for a potential use in cancer diagnosis and prognosis, and to eventually monitor the tumour response to therapy. Finally, these data have greatly inspired the development of various strategies to target telomere and telomerase for cancer therapy.

## 3. Anti-telomere and anti-telomerase drugs

### *3.1. Targeting hTERT*

Targeting the hTERT catalytic sub-unit as anticancer therapy is theoretically tumour-specific and might be moderately

toxic due to its expression spectrum in tumour and highly proliferating cells compared to other normal cells. Various nucleoside triphosphates or non-nucleoside reverse transcriptase inhibitors are under investigation, and together with some recently developed antisense strategies these represent interesting anti-hTERT candidates for clinical drug development (Table 2).

### 3.1.1. Nucleoside inhibitors

Nucleoside analogs such as AZT (3′azido-3′deoxythymidine) are small molecules with reverse transcriptase inhibitory effect, and were tested against telomerase as early as a decade ago. These analogs block the incorporation of dNTPs into the neosynthesized DNA during the reverse transcription activity. In early studies, AZT was able to partially reduce the telomerase activity, but the cells showed only some weak proliferative impairment. Nevertheless, a transient reduction of telomere length was observed [101,102]. Other nucleoside analogs such as derivates of AZGTP (7-deaza-2′-deoxygunosine 5′-triphosphate) might show a stronger inhibitory potential [103]. Nevertheless, reported results are inconsistent and there might be some indirect effects on apoptosis such as incorporation of the nucleoside analogs into mitochondrial DNA causing the depletion of mtDNA and mitochondrial toxicity. Therefore, more arguments are needed before nucleoside inhibitors might have applications in the clinic as efficient anticancer molecules.

### 3.1.2. Non-nucleosidic inhibitors

Non-nucleoside inhibitors are compounds that inhibit the telomerase through binding to the active site of the reverse transcriptase enzyme. These small molecules have shown potential to inhibit the telomerase activity and cause progressive telomere shortening in pharmacological screening programs, as for instance the isothiazolone derivative TMPI [104], the rhodacyanine FJ5002 [105], and the BIBR 1532 [92].

One of the most promising molecules is probably the BIBR 1532, which is a non-nucleoside inhibitor that does not affect other DNA and RNA polymerases than the telomerase. BIBR 1532 is a very specific drug and a non-competitive inhibitor of the telomerase enzyme, suggesting that the binding site is different from the DNA primer and nucleotide binding sites. Further studies have revealed that BIBR 1532 interferes with the processivity of the telomerase [106]. Its use in different cancer cell lines from four different tumour entities led to progressive telomere shortening followed by senescence-like growth arrest, and further induced a significant decrease of the tumourigenic potential in vivo [92]. The ability of BIBR 1532 to inhibit telomerase has recently been confirmed by others and further completed by the discovery of other equally potent analogues [107]. Nevertheless, the mechanism of BIBR 1532 remains unclear. New findings suggest that high doses of BIBR 1532 lead to a selective cytotoxicity in leukaemia cells with telomerase-independent telomere erosion and loss of TRF2 and increased phosphorylation of p53 [108]. Interestingly, no such effect was observed in CD34+ stem cells, and normal fibroblasts with overlong telomeres were primarily resistant to the treatment. Such selective and rapid telomeric cytotoxicity might be a very useful approach in anti-cancer drug development and further in vivo studies are greatly awaited.

### 3.1.3. Antisense technology

The use of standard oligodesoxynucleotides have shown somehow disappointing results with limited stability and bioavailability, but a recent study developed a new powerful hTERT antisense oligodeoxynucleotides able to specifically inhibit telomerase activity and cell growth in bladder cells [109]. Synergism between this strategy and conventional chemotherapy (mitomycin C, cisplatin and gemcitabine) has also been shown with a specific 1.3- to 3.0-fold increase of the apoptosis rate in transitional cell carcinoma cells from bladder after combined treatment [110]. However, developing peptide nucleic acids, PNAs (analogs of DNA and RNA) that are resistant to the degradation of exo and endonucleases might be even more promising. PNAs are able to act through a specific inhibition of telomerase and they also fit the criteria of telomerase inhibition selectivity. Unfortunately, the low membrane permeability of PNAs is a major obstacle to obtain optimal cell delivery. However, hTERT-PNAs have been tested on prostate tumour cell lines by a photochemical internalization method [111]. This internalization technology was more efficient than an HIV-Tat protein-based approach. After light-exposure, the cells showed marked inhibition of telomerase activity and reduced cell survival compared to cells treated with hTERT-PNA alone. The therapeutic potential of PNA antisense strategies is very promising, but studies proving the efficiency of antisense-mediated hTERT mRNA inhibition with eventual tumour regression should first be clearly demonstrated in animal models.

There are still few studies that have taken advantage from the recently developed RNAi technology to target telomerase activity using stable short-interfering RNA (siRNA). Kosciolek et al. showed that telomerase activity could be inhibited by siRNAs targeting telomerase components. In their study, a transient and modest inhibition was shown in a variety of carcinoma cell lines (colon, brain, lung, and skin). Inhibition was also shown in cell lines of mesodermal origin (osteosarcoma and fibrosarcoma), although inhibition appeared to be of shorter duration than in the carcinoma cell lines tested [112]. Other investigators have also explored such strategies to target telomerase. In a recent report in Chinese, Lu XD et al. seemingly obtained the inhibition of telomerase activity in a hepatocellular carcinoma cell line by siRNA, resulting in a remarkable loss of hTERT protein, a 76% loss of telomerase activity, and an apoptotic rate significantly higher than in control cells [113]. Interestingly, another Chinese study claims a reduction of tumour size by RNAi technology after transplantation of a hepatocellular carcinoma cell line in nude mice [114]. Confirmation of these results is needed, but it seems clear that RNAi technology has a high potential to

Table 2
Overview of anti-telomerase and anti-telomere strategies and tumour models tested

| Target | Class | Name | Mechanism | Pharm. level | Tumour model/organ site tested | References |
|---|---|---|---|---|---|---|
| hTERT protein sub-unit | Enzyme inhibitors/small molecules | AZT | Blocks dNTP incorporation into DNA | in vitro | Breast, leukaemia, and cervical cancer cell lines | [101–103] |
| | | TMP, FJ5002, BIBR1532 | Specific inhibition of the active site | in vitro | Breast, lung, prostate, leukaemia, fibrosarcoma, hepatoma, and cervical cancer cell lines | [92,104–108] |
| | | | | in vivo | Fibrosarcoma xenografts | |
| | Antibiotics | Distamycin derivatives (MEN10716) | Catalytic inhibition of hTERT | in vitro | Melanoma and lung cancer cell lines | [124] |
| | Vitamins | 1,25-Dihydroxy Vitamin $D_3$ | Catalytic inhibition of hTERT | in vitro | Ovarian cancer cell lines | [123] |
| hTERT mRNA | Antisens molecules | DNA, PNA, siRNA, Ribozymes | Plasmid- or not plasmid-based expression of complementary antisens RNA blocking/degrading hTERT mRNA | in vitro | Breast, lung, prostate, colon, bladder, melanoma, brain, ovarian, endometrial carcinoma, osteosarcoma, fibrosarcoma, and hepatocellular carcinoma cancer cell lines | [109–117] |
| | | | | in vivo | Hepatocellular carcinoma xenografts | |
| hTR (RNA component of telomerase) | Antisens molecules | DNA (DNS, GRN163), PNA, siRNA, Ribozymes | Plasmid- or not plasmid-based expression of complementary antisens RNA blocking/degrading hTERT mRNA | in vitro | Breast, lung, prostate, colon, bladder, melanoma, glioma, gastric, myeloma, lymphoma, epidermoid carcinoma, cervical carcinoma, and gastric cancer cell lines | [125–142,145–149] |
| | | | | in vivo | Prostate, glioma, bladder, melanoma, lymphoma, and myeloma xenografts | |
| | Mutated polypeptides, deletion-spliced mRNA isoforms (plasmid based) | DN-hTERT, hTERTalpha | Dominant negative action on telomerase activity (sequestering of hTR) | in vitro | Breast, lung, colon, melanoma, leukaemia, epidermoid carcinoma, kidney, ovarian, melanoma, and fibrosarcoma cell lines | [83,93,95,119–122] |
| | | | | in vivo | Ovarian xenografts | |
| Transcription factors of hTERT | Small molecules, plasmid -based expression of peptides, hormone receptor agonists | Porphyrins (TMPyP4), Tyrphostins (AG825), Nmi protein, BRCA1, DN-ER81, tamoxifen, ATRA agonists (Am580, CD3640) | Inhibition of c-MYC, ER81, estrogens, HER/Neu, and trans-retinoic acid receptors, resulting in downregulation of hTERT | in vitro | Breast, prostate, leukaemia, pancreatic, cervical carcinoma, and endometrial cancer cell lines | [157–159,169–171] |
| | | | | in vivo | Breast and prostate xenografts | |
| Chromatin in the hTERT promoter region | Demethylating agents, histone deacetylase inhibitors | 5-Azacytidine, Tricostatin A | Change in chromatin structure inhibiting hTERT transcription | in vitro | Prostate, colon, neuroblastoma, and cervical cancer cell lines | [162–164] |

| | | | | | | |
|---|---|---|---|---|---|---|
| Post-translational modifications of telomerase | Antibiotics | 17-Allylamino 17-demethoxygeldanamycin | Inhibition of hsp90 chaperone function impairing telomerase maturation | in vitro | Melanoma | [174] |
| Telomerase co-factors | Antibiotics, 3-PI-kinase-inhibitors, PKC-inhibitors, diterpenoid-quinine | Novobiocin, Wortmannin, bis-indolylmaleimide, salvicine | Dephosphorylation of telomerase co-factors or hTERT itself | in vitro | Breast, lung, leukaemia, melanoma, kidney,, nasopharyngeal carcinoma and cervical cancer cell lines | [175,177–182,210] |
| Telomere structure | Antisens molecules | siRNA | Plasmid based hTR-mediated introduction of mutations into the telomeric DNA | in vitro | Breast, Prostate, colon, melanoma, and bladder cancer cell lines | [143,144] |
| | | | | in vivo | Bladder xenografts | |
| | G-quartet interactive ligands | BRACO-19, quinoline triazines (115405, 12459), 2-6-pyridine dicarboxamides (831A, 832A, 307A, 360A), Telomestatin (SOT-095), PNA, cisplatinum | Binding to telomeric DNA impairing its structure | in vitro | Breast, lung, colon, leukaemia, hepatoma, melanoma, glioma, vulval, uterin carcinoma, myeloma, and cervical cancer cell lines | [119,141,142,190–194,196–201,205,206] |
| | | | | in vivo | ALT-cells<br>Vulval and uterin carcinoma xenografts | |
| Telomere structure? | Anthracyclines, alkaloids (topoisomerase-II inhibitors) | Doxorubicin, etoposide | Unknown (changing telomere structure or integrity) | in vitro | Gastric cancer cell lines | [207,208] |
| Telomere-associated proteins | Polypeptides (plasmid-based), catechols, PARP-inhibitors | DN-TRF2, MST-312, 3-aminobenzamide | Inhibition of TRF2 or tankyrase, deprotection of telomere structures | in vitro | Breast, cervical carcinoma, fibrosarcoma, and osteosarcoma | [33,218] |
| Tumour-specific antigens such as hTERT | Adaptive T-cell immunotherapy, cellular immunotherapy vaccination | hTERT-derived peptides, transfected CTLs or dendritic cells | Generation of cytotoxic T-lymphocytes or specific B-cells | in vitro | Breast, prostate, melanoma, leukaemia, renal, lymphoma, and myeloma cancer cell lines | [221–229] |
| | | | | in vivo, phase I clinical trials | Renal, prostate, and melanoma animal models<br>Trials in breast, prostate and renal cancer | |
| Pro-apoptotic genes, oncolytic viruses, prodrugs | Gene therapy | Plasmids controlled by hTERT promoter | hTERT positive cancer cells activates anti-tumour strategies | in vitro | Breast, lung, colon, gastric, pancreatic, hepatocellular carcinoma, glioma, cervical, and fibrosarcoma cancer cell lines | [235–256] |
| | | | | in vivo | Gastric, ovarian, thyroid, and fibrosarcoma animal models | |

effectively target telomerase and become an interesting candidate for further drug development.

#### 3.1.4. Ribozymes

Hammerhead ribozymes are antisense RNAs possessing specific endoribonuclease activity that catalyzes the hydrolysis of specific phosphodiester bonds, which eventually results in a cleavage of the targeted RNA sequences. Theoretically, they have a large pharmacological potential such as high stability (chemical modifications), bioavailability (uptake by conjugated proteins or lipids), and specificity (sequence specific). Assays of degrading the mRNA of the catalytic telomerase subunit hTERT by ribozymes have been successful in various tumour cell lines such as endometrial, breast and ovarian carcinoma [115–117]. These studies resulted in a loss of telomerase activity, inhibition of cell proliferation, and apoptosis. Nevertheless, telomere shortening was not always demonstrated and further studies using ribozyme technology against hTERT mRNA are needed. Moreover, a supplemental difficulty with the antisense strategy in general, might be the presence of several versions of hTERT mRNA by alternative splicing. Indeed, it has been shown that the hTERT transcript has at least six alternative splice isoforms [118].

#### 3.1.5. Dominant negative hTERT

By inducing specific alterations of amino acids, some mutant hTERT proteins become catalytically inactive, but remain able to sequester the RNA component hTR. Two studies have shown in vitro the capacity of DN-hTERT constructs to inhibit telomerase activity in immortalized and tumour cells [93,95]. Comparing different cell lines, dependence between the time before crisis and initial telomere length was demonstrated. Interestingly, Hahn et al. also showed potential of this method in vivo by injecting ovarian cancer cells into immunodeficient nude mice [93]. The cells containing the wild-type hTERT and control vectors readily produced tumours whereas the cells with the DN-hTERT failed to form tumours. It is also interesting that DN-hTERT constructs sensitise the tumour cells to other antineoplastic therapies, such as cisplatin, docetaxel, etoposide, ecteinascidin-743, temozolomide and imatinib [119–122]. However, potential problems to be solved before treating patients with the dominant negative approach are how to ensure a proper cell delivery (target cancer cells with efficient uptake of the vector) and overcome risk factors related to gene therapy. Thus, while appealing, this approach is still far from entering the clinical arena.

#### 3.1.6. Other hTERT inhibitors

Other molecules (notably antibiotics) have shown the capacity to inhibit the hTERT sub-unit and have been tested for drug development. Recently, Jiang et al. [123] showed the capacity of 1,25-dihydroxyvitamin $D_3$ to induce apoptosis in ovarian cancer cells. The apoptosis was not due to transcriptional repression through the Vitamin D response element present in the 5′ regulatory region of the hTERT gene, but a significant decrease of the stability of hTERT mRNA was observed. However, no loss of telomere length was found, and it remains unclear if the telomerase down regulation induced itself cell apoptosis through modulation of telomere integrity or if the apoptosis was induced by a telomere-independent pathway.

Another candidate drug called MEN 10716 (a distamycin derivative) is also a candidate as telomerase inhibitor. Effectively, it has been shown in vitro that the abrogation of telomerase activity by MEN 10716 can affect cell proliferation even through pathways that are not dependent on telomere erosion [124]. However, further understanding of the specific mode of action of these molecules and in vivo validation are needed before any direct clinical application can be developed.

### 3.2. Targeting hTR

Targeting the RNA hTR is a strategy that aims at blocking the access of telomerase to its template RNA resulting in inhibition of telomerase activity. Another promising approach is to employ mutant hTRs that introduce mutations into the telomeres impairing indirectly the structure of telomeres (Table 2).

#### 3.2.1. Antisense oligonucleotides and siRNA

Strategies using transfection of antisense expression vectors against hTR have been developed. Most studies have been able to induce a decrease in telomerase activity and demonstrate a progressive cellular senescence in the targeted cells. Nevertheless, the stability and bioavailability of such molecules have been focused on through some very challenging research. Significant improvements of PNA uptake have been observed by generating PNA–DNA complexes or by conjugation with transport peptides [125,126]. Further, strategies using cationic peptides at the N-terminus of PNA molecules demonstrated an enhanced inhibition of telomerase activity in different cell lines [127], and more recently, PNA–DNA heteroduplexes were introduced by lipid-mediated transfection into a human gastric cell line significantly inhibiting telomerase activity and cell growth [128].

Moreover, studies using N3′ → P5′ thio-phosphorothioate oligonucleotides (NPS) have shown a potential therapeutic effect in human breast carcinoma cell lines [129]. The long-term treatment resulted in gradual telomere shortening, cellular senescence, and widespread apoptosis. After sequence and length optimization, the bioavailability was further increased and led to a very potent hTR antagonist called GRN163 [130]. GRN163 is able to inhibit telomerase activity and provoke senescence and apoptosis after progressive telomere shortening in various cell lines, including human multiple myeloma (MM) and non-Hodgkin lymphoma (NHL) cell lines [131,132]. Interestingly, GRN163 significantly suppressed tumour growth in several mouse xenograft models such as human prostate cancer, glioblastoma, MM and NHL [131–133]. These impressive results

demonstrate clearly that GRN163 has a significant potential for development as an anticancer agent, but it also remains a plasmid-based approach.

Similarly, other investigators have used alternative types of RNA oligonucleotide constructs to target the template region of hTR, such as antisense phosphorothioate oligonucleotides (S-ODNs) or as 2′-*O*-(2-methoxyethyl) RNAs (2′-MOEs) [134–136]. The introduction of 2′MOEs into the human prostate cell line DU145 led to telomerase inhibition for 7 days and progressive telomere shortening with eventual apoptosis of the treated cells with critically short telomeres [136].

Another promising candidate is the 2′–5′ oligoadenylate antisense anti-hTR (2-5A). Repeated injections of liposomes containing 2-5A into mice demonstrated a significant anti-tumour effect. The expression of such oligoadenylate oligonucleotides will normally lead to the activation of RNAaseL, which is responsible for the degradation of single-stranded RNA, a process taking part of a normal autonomous antiviral system leading to apoptosis in human cells. Several xenograft models of glioma and bladder cancer treated with 2-5A, showed tumour shrinkage specifically due to apoptosis induction [137–139]. Additionally, synergism between this approach and conventional cytotoxics such as cisplatin were also reported [140–142], which places 2-5A as a serious candidate for further development.

Targeting hTR by siRNA has recently been tested with success, both in vitro and in vivo by Elizabeth Blackburn's team [143]. Using human melanoma, breast, colon, prostate, and bladder cancer cell lines, they developed a hairpin short-interfering RNA that specifically targets the endogenous wild-type hTR template region, but spares a mutant hTR introduced concomitantly into the cancer cells by a lentivirus. The mutant hTR makes the telomerase add mutant DNA to the telomeres, which by itself is able to cause a significant cell growth inhibition and apoptosis [144]. The combination of both anti-wild-type hTR siRNA and mutant hTR, acts synergistically to kill telomerase positive cancer cells. Tumour growth and progression were also significantly decreased in the mouse xenograft model [143]. The requirement for up-regulated hTERT, the rapidity of the killing, the failure to see any resistant cell subpopulations, and the lack of dependence on p53, initial telomere length, or progressive telomere shortening suggest that this strategy targeting both telomerase and the telomere structure is an attractive candidate for further development as anticancer treatment.

#### 3.2.2. Ribozymes

As already mentioned, antisense strategy has showed significant progress over the last years, and has elegantly been enriched by the ribozyme technology. Ribozymes used against hTR have given specific telomerase inhibition and cell growth delay in several cell line studies, but effective apoptosis or reductions in telomere length have not fully been observed [145–147]. Nevertheless, a recent study demonstrated a clear attenuation of telomerase activity by hammerhead ribozymes targeting hTR that was proved to effectively induce apoptosis in human breast tumour cells [148]. More interestingly, using a melanoma tumour-bearing mouse model, a systemic distribution of EBV-based plasmids produced sustained levels of ribozyme expression targeting the mouse telomerase RNA (mTER) [149]. The latter study successfully reduced both telomerase activity and metastatic progression in vivo. Even if murine models are probably not optimal because of their much longer telomere lengths compared to humans, this work highlights the potential of plasmid-based anti-hTR ribozymes as anticancer therapy. But again, a direct clinical application might be limited by issues around gene therapy.

### 3.3. Targeting regulatory mechanisms of telomerase at the transcriptional or post-transcriptional level

Regulation of the expression of telomerase is controlled both at the transcriptional and post-transcriptional levels. Hence, targeting regulatory factors of telomerase has been evaluated in several studies.

#### 3.3.1. Transcriptional level

An important regulatory mechanism is the effect of different transcription factors on the promoters of hTR and hTERT. A large number of transcription factors acts on the promoter of hTERT, like SP1 [150], c-MYC [151], the estrogen receptor [152], E2F-1 [153], WT-1 [154], and MZF-2 [155] and have been reviewed elsewhere [156]. Particularly, the transcription factor c-MYC, which is closely connected to the proliferation behaviour of cells, is an interesting candidate target for the inhibition of telomerase activity. The cationic porphyrin, TMPyP4, which downregulates c-MYC and therefore hTERT expression, has been described as an inhibitor of tumour growth in vivo [157]. Further, transcriptional down-regulation of hTERT expression via a complexing of c-MYC has also been evaluated, such as the c-MYC complexing with BRCA1 protein and N-MYC-interacting protein (Nmi) that inhibits c-MYC-induced hTERT promoter activity in breast cancer [158]. Moreover, the oncoproteins HER2/Neu, RAS, and RAF, stimulate hTERT promoter activity via the ETS transcription factor ER81 and ERK mitogen-activated protein (MAP) kinases [159]. Suppressing the phosphorylation of ER81, or mutating its binding sites in the hTERT promoter was able to render the hTERT promoter unresponsive to HER2/Neu. Exploiting this knowledge, it was shown that a plasmid-based introduction of a dominant-negative ER81 or the inhibition of the HER2/Neu receptor in breast cancer cells significantly attenuates telomerase activity [159].

Demethylating agents are able to activate genes by reversing the hypermethylation of silenced gene promoters. Demethylation has been related to altered expression of tumor suppressor genes (Herman 1994–1996). In contrast, a positive correlation has been observed between hypermethylation of the CpG island of the hTERT promoter and mRNA expression and telomerase activity in a multitude of cancer cell

lines and confirmed in tissues [160,161]. Interestingly, the inhibitory effect of the demethylating agent 5-azacytidine on telomerase activity and hTERT expression has been shown in several cancer cell lines, including HeLa, colorectal, neuroblastoma and prostate cells, with reduced cell growth in the latter cells [162,163]. However, the correlation between hTERT demethylation and gene repression is opposite to the current dogma of regulation by DNA methylation. Indeed, further analysis in prostate cells revealed that 5-azacytidine reactivated p16 expression and repressed c-Myc expression in one of the cell line tested. Therefore, 5-azacytidine might in fact inhibit telomerase activity via a transcriptional repression of hTERT, in which p16 and c-MYC could play a central role [163]. Of course, another challenge of demethylating agents or even of histone deacetylase inhibitors such as tricostatin A, which has also been able to inhibit hTERT mRNA expression in prostate cells [164], is their lack of gene specificity, and they therefore remain controversial.

Several hormones play also a role in the upstream signalling of the hTERT transcriptional activation, such as estrogen and progesterone [152,165,166], and retinoids [167,168]. Tissue-dependent expression might be based on hormone-dependent regulation of telomerase activity as suggested by the fact that tamoxifen, an antiestrogenic agent, is able to block hTERT transcription and cell growth in estrogen-receptor positive breast tumour cells, but stimulates the transcription of hTERT in endometrial cancer cells [169,170]. Nevertheless, given the tissue-specific expression of nuclear retinoic-acid receptors (RAR), a tissue-selective therapy targeting telomerase in tumour cells by synthetic agonists such as a dual-liganded retinoid-X receptor (RXR) and RARalpha has been proposed [171]. Interestingly, the latter study demonstrated recently that the cross-talk created between RARalpha and the retinoid-X receptor when dual-liganded to their respective agonists, results in a strong synergistic downregulation of hTERT and subsequent cell death.

#### *3.3.2. Post- transcriptional level*

Splicing of the hTERT mRNA is an important regulatory mechanism and one particular splice product has been found to be a dominant negative inhibitor of telomerase activity [83]. Furthermore, regulation of growth and telomerase activity in skin cells has been uncovered suggesting a dual c-Myc-dependent inhibition and alternative hTERT splicing [172]. Telomerase is also associated with several accessory proteins like TEP-1, p23, hsp90 (Fig. 2) and interacts with modifying enzymes like phosphatase A, protein kinase C, Akt-kinase and others that play important roles for assembly and function of the holoenzyme or regulate post-translational modification. In particular, blocking the hsp90 chaperone function leads to inhibition of telomerase assembly [76,173–175]. Recently, Villa et al. [174] investigated the effect exerted by the ansamycin antibiotics geldanamycin (GA) and 17-allylamino,17-demethoxygeldanamycin (17-AAG), two well-known inhibitors of the hsp90 chaperone function, on telomerase activity in human melanoma cells. Exposure to GA and 17-AAG induced early inhibition of telomerase activity, followed by inhibition of cell proliferation. Interestingly, like a predictive marker, the basal level of telomerase activity seems to have played a role in the cellular response to ansamycin, as the cells with markedly lower telomerase activity showed an apoptotic response almost two-fold compared to parental cells.

Recently, it has been shown in human ovarian cancer cells, that 17-beta-estradiol induces telomerase activity by post-transcriptional regulation via Akt-dependent phosphorylation of hTERT [176]. Thus, the phosphorylation of Akt might be a key event in the induction of telomerase activity in cancer cells. As phosphorylation is essential for the functioning of the enzyme, one can imagine many strategies to influence telomerase activity via such co-factors. Taking advantage of such knowledge, several studies have shown that the activation of protein phosphatase 2A (PP2A) decreases telomerase activity in tumour cells like melanoma and breast, whereas okadaic acid, an inhibitor of PP2A, stimulates both hTERT phosphorylation and telomerase activity [175,177–179]. Furthermore, using PKC inhibitors such as bis-indolylmaleimide I, a significant inhibition of telomerase activity has been observed in nasopharyngeal carcinoma cells [180,181] and in cervical cancer cell lines [182].

Finally, the assembly of the telomerase components seems to happen at the nucleolar level [78,183,184], and it is known that telomerase interacts with nucleolin [185]. Further, the loss of the extreme N-terminal domain of hTERT (1–15), which targets the nucleolar localization of the protein, hampers the full telomerase function [186]. Additionally, the nucleolar shuttling seems to be impaired in tumour and transformed cells [187]. Clearly, the subcellular localization of telomerase is an important mechanism of functional regulation, and needs to be kept in mind when designing new anti-telomerase and telomere therapies.

### *3.4. Targeting the telomeres and associated proteins*

To target directly the element that seemingly forces cells into crisis, the length of telomeres, has largely enriched the development of anticancer candidates. Emerging evidence proposes that it is the shortest telomeres rather than average telomere length that cause chromosome end fusions and apoptosis in telomerase-inhibited cells [188]. However, it is still not fully known what mechanisms and molecular actors are implicated in the telomere dysfunction related cell crisis.

#### *3.4.1. G-quadraduplex DNA-interactive compounds*

The G-rich single stranded telomere overhang is able to fold back on itself in vitro to form 4-stranded G quadruplex (or tetraplex) structures, which are poor substrates for telomerase. A second model, equally evidenced in vitro by Titia de Lange's laboratory, suggests that the telomere repeated sequence folds back on itself to form a duplex loop structure termed T-loop [17]. Nevertheless the structure of human telomeres in vivo is not fully determined, and

it remains unclear whether the chromosome ends actually form T-loop structures, G quadruplexes, or other structures in vivo (Fig. 1). However, some very potent telomere targeting agents include G quartet interactive agents, also called "G4" molecules. The principle of action of G4 molecules is to stabilize the G quadruplex structures, presumably by intercalation, and therefore block the telomerase during the elongation step. A number of G-quartet stabilizing agents are currently under investigation. They belong to different molecular families such as ethidiums, dibenzophenanthrolines, triazines, acridines or pentaoxazoles. However, porphyrin, acridine, anthraquinones and fluorenone-based compounds are until now the most promising ones.

In particular, trisubstituted acridine derivatives are very potent and selective telomerase inhibitors [189]. The G-quadruplex interacting agent, 9-[4-(*N*,*N*-dimethylamino)-phenylamino]-3,6-bis(3-pyrrolodinopropionamido) acridine, also called BRACO-19, represents one of the most potent cell-free inhibitors of human telomerase yet described (50% inhibitory concentration of only 115 nM). A major advantage of BRACO-19 is to avoid acute nonspecific cytotoxicity at equivalent concentrations required to completely inhibit telomerase activity, at least in human breast cancer cells, and a marked reduction in cell growth has been observed in these conditions [190]. Interestingly, in vivo studies using non-toxic doses of BRACO-19 in mice previously treated with paclitaxel and bearing advanced stage human vulval carcinoma xenografts, induced a significant increase in antitumour effect compared to mice treated with paclitaxel alone [190]. Furthermore, BRACO-19 treatment of uterus carcinoma xenografted mice has been able to inhibit 96% of tumour growth, and the treatment was accompanied by loss of nuclear hTERT expression and telomere dysfunction [191].

The quinoline-substituted triazines, called 115405 and 12459 are two other potent G-quadruplex-stabilizing compounds with an IC50 for in vitro telomerase inhibition of 41 and 130 nM, respectively [192]. Ligand 115405 induced a dose-dependent decrease in telomerase activity in treated cells, and both ligands had antiproliferative properties. In particular, the ligand 115405 was active on several human cancer cell lines, but also on immortalized human cell lines including the ALT cell line GM847DM, an SV40 immortalized lung fibroblast cell line, and a telomerase immortalized fibroblast. It was further demonstrated that telomerase activity is not the main target of the 12459 ligand, and that resistance to antiproliferative activity might be associated with hTERT functions and telomere capping alteration [193,194]. Further studies have suggested that telomerase downregulation of 12459 is mediated by hTERT RNA alternatively spliced isoforms through stabilization of quadruplexes located in the hTERT intron 6 [195].

Recently, new G4-ligands such as 2,6-pyridine-dicarboxamide derivatives have shown massive apoptotic effects using low concentrations in glioma cells related to cell cycle alterations and telomere instability rather than telomere shortening [196]. The telomere instability involved telomere end fusions and anaphase bridges. Interestingly, these molecules also had antiproliferative effects in a cell line with ALT-mechanism.

Another inhibitor candidate is telomestatin (SOT-095), which is a natural product from Streptomyces anulatus [197]. Recently, it has been demonstrated that telomestatin has an important effect on the conformation of intracellular G-overhangs [198]. The competition experiments indicated that telomestatin strongly binds in vitro and in vivo to the telomeric overhang impairing its single-stranded conformation, hence progressively reducing the G-overhang size with concomitant delayed loss of cell viability. Telomestatin has recently been tested on myeloma cells [199] and was able to induce an inhibition of telomerase activity, reduction in telomere length, and apoptotic cell death. Remarkably, no changes were seen in the expression of genes involved in cell cycle, apoptosis, DNA repair, or recombination, suggesting that telomestatin exerts its antiproliferative and proapoptotic effects specifically through the telomeric pathway. Telomestatin has further been tested on leukemia U937 and NB4 cells [200] where it inhibited telomerase activity followed by telomere shortening. Enhanced chemosensitivity to daunorubicin and cytosine-arabinoside was also observed. Interestingly, in the same study, telomere shortening associated with apoptosis by telomestatin was demonstrated in freshly obtained leukemia cells from acute myeloid leukemia (AML) patients.

Other approaches use the advantages of peptide nucleic acids (PNAs) to target the telomeric G-rich strand, and their efficacy to reverse the immortality of transformed human fibroblasts has been tested [201]. The experiments resulted in increased cell death rate by apoptosis. Further, a combination of this anti-telomere PNA inhibitor with a PNA that additionally blocked telomerase activity resulted in a nearly complete inhibition of colony growth, induction of apoptosis, and reduction in telomere length [201]. These observations indicate that an enhanced efficacy for therapeutic approaches can be reached by targeting multiple, distinct mechanisms of telomere maintenance.

### *3.4.2. Conventionnal cytotoxic compounds*

Cisplatin (CDDP) is a well-known alkylating agent used in numerous chemotherapy regimens to treat cancer. Both intra- and interstrand covalent bridges blocking the transcription and/or replication of DNA assure the cytotoxic effect of CDDP. Its effect on telomeric DNA is less understood, but CDDP has clearly a high affinity for the nucleophilic sites of guanidine and adenine nucleotides, and therefore, intrastrand covalent bonds between two consecutive guanosine bases and/or interstrand bounds are predicted in the telomeric TTAGGG repeat region. Effectively, it has been demonstrated in vitro that CDDP not only forms 1,2-intrastrand adducts on double stranded telomere sequences [202], but also cross-links adenines and guanines brought sufficiently close to each other on the G-quadruplex structure of the single-stranded

telomere overhang [203,204]. This suggests that CDDP-mediated cross-linking of the G-quadruplex structures could prevent structured single-stranded telomere sequences from unfolding and might therefore inhibit telomerase activity. Moreover, CDDP has been reported as an inhibitor of telomerase activity in human testicular cancer cells and a significant loss of telomere length has been observed in CDDP-treated HeLa cells and hepatoma cells [205,206]. Interestingly, synergism between CDDP and telomerase inhibition by the 2′,5′-oligoadenylate linked anti-hTR oligonucleotide has been observed both in vitro and in animal models [141,142]. Further, telomere dysfunction through downregulation of the c-Myc gene increased the sensitivity of melanoma cells to CDDP and the novel anti-cancer drug ecteinascidin-743 [119]. Therefore, sustained efforts to better understand the loss of telomere length due to CDDP are highly warranted.

Other clinically used cytotoxic compounds have shown to have some effect on telomere length or telomerase activity, such as doxorubicin in gastric cell lines [207]. Direct telomere cleavages by topoisomerase II activity following treatment with etoposide (VP16) have also been reported [208], and synergism between etoposide and telomerase inhibition has been observed in immortalized fibroblasts and in human breast cancer cell lines. Other cytotoxic drugs might have surprising and unexpected effects on telomeres. A recent example is the diterpenoid quinine called salvicine, which is a novel DNA topoisomerase II inhibitor that exerts its antitumour effect by trapping the enzyme-DNA cleavage complexes [209]. Salvicine was shown to indirectly inhibit telomerase activity in the lung carcinoma cell line A549 without affecting the expression of hTERT or hTR mRNA. But most interestingly, salvicine shortened the telomere length by 30% after only 4 h of exposure [210]. Further studies should permit to distinguish between specific and unspecific drug effects at this level and clear out the mechanism of such dramatic replication-independent telomere loss. However, it illustrates how a combination between targeted and non-targeted therapy might give unexpected synergisms. For instance, it could be interesting to evaluate the effect of coupling a salvcine treatment with a highly specific telomerase inhibitor on different cell lines.

#### *3.4.3. Targeting telomere-associated proteins*

Targeting telomere-binding proteins leads to deregulation of telomere maintenance. It has been proposed that the uncapping of only one or some few telomeres might signal cell cycle arrest and apoptosis in human cancer cells [144]. Karlseder et al. have already shown that the introduction of a dominant negative TRF2 into cancer cell lines results in telomere shortening and rapid p53-dependent apoptosis that is not dependent on initial telomere length [33]. Further, it has been proposed that TRF1 acts as a *cis* inhibitor of telomerase activity [23]. According to this model, targeting TRF1 could be a potential target for anticancer drug development. However, a recent study using conditional TRF1-deficient mouse ES cells showed that TRF1 is important for telomere length regulation but also essential for normal cell growth, telomere structure and chromosomal stability [211]. Therefore, diminishing telomere function by targeting telomere-associated proteins does not necessarily target exclusively cancer cells and may even promote genomic instability in normal cells contributing to increased cancer incidence. Indeed, recently published experiments have shown that cycling human fibroblasts exhibit weak expression of hTERT and telomerase activity. This low level of activity has essential biological consequences even if this telomerase expression is insufficient to maintain overall telomere length in normal cells [212]. Additionally, it has been shown that telomere length is decreased in tumours or pre-invasive lesions as compared to normal tissues [213–217], hence telomeres may possibly have a different structure in normal cells compared to tumour cells. Changes in the structure of telomeres are already essential in the process of senescence [26]. Clearly, approaches targeting the telomeric capping function raise completely new challenges. It is, however, tempting to speculate that such strategies might become very tumour-specific if properly coupled with a vector system under an hTERT promoter control. Combining this approach with for instance Karsleder's mutant TRF2 could theoretically be a way to target cancer cells. Interestingly, important studies from Seimiya and colleagues have recently shown that combinations of tankyrase and telomerase inhibitors at nontoxic doses may also be an effective anticancer therapeutic approach [218]. The authors exploited the knowledge of tankyrase action by asking whether manipulating the ability to recruit telomerase to act on telomeres could prevent the maintenance of a new telomere equilibrium maintenance length in tumour cells. First, a nontoxic dose of the telomerase inhibitor MST-312, a chemical derivative of a component of green tea, partially inhibited telomerase and shortened the telomere lengths from 5 kb to a new stable length of 4 kb. Then adding 3-aminobenzamide (a general PARP inhibitor that inhibits tankyrase) to the previously MST-312-exposed cells caused telomere shortening again until the cells entered crisis and died, whereas no such effect was seen in control cells. Clearly, advances in strategies targeting telomere-binding proteins are underway and might be powerful anticancer candidates.

## 4. Immunotherapy

The asset for antitumoural vaccination emerged from the discovery that almost all tumours express antigens. In fact, human tumour-associated-antigens (TAA) have now been characterized in most malignancies, particularly in melanoma. Such studies [219,220] have shown in cancer patients that TAA involves the generation of specific cytotoxic T lymphocytes (CTLs) that recognize peptides derived from these antigens. These specific CTLs can destroy the corresponding tumours in vitro. Therefore, immunization with TAA recognized by tumour specific CTLs, also called

adoptive T cell therapy, should represent an effective strategy for cancer immunotherapy.

The catalytic subunit of telomerase hTERT is a promising candidate as a universal TAA and it can be processed by the proteosomes and be presented on the surface of cancer cells in an MHC context as an antigen recognized by CTLs. Several working groups have isolated hTERT-specific CTLs able to lyse cancer cell lines and tumours in a telomerase and MHC-restricted fashion. hTERT-derived peptides can be identified for several of the prevalent MHC haplotypes. Vonderheide et al. [221] identified an HLA-A2 binding hTERT-derived peptide generating a CTL line that kills hTERT positive tumour cells in vitro from a broad range of human tumours. Other studies discovered new hTERT-derived peptides that were also able to generate specific CTLs that kill tumour cells in vitro [222–225]. In addition, Nair et al. [226] used hTERT-RNA transfected DCs to stimulate hTERT specific CTLs in vivo, which successfully killed renal and prostatic human tumour cells in a mouse model. Further, it has recently been demonstrated that hTERT-transduced CTLs inhibit the growth of human melanoma in nude mice [227]. Following these very promising preclinical results, several phase I trials using hTERT as a TAA have started. The first to be published evaluated 10 patients with metastatic renal cancer who had received RNA-transfected DCs [228]. After the treatment, TAA-specific CTLs, including hTERT were detected in six patients. No specific CTLs against self-antigens expressed by normal renal tissue were characterized. As for the clinical response, seven patients were alive after a mean follow-up of 19.8 months.

Another method of active immunotherapy against hTERT peptides has recently been developed (Millard et al., Proc. ASCO 2004, Abstract 2519). Here, transfected B-lymphocytes with a plasmid that incorporates two HLA-A2 restricted hTERT peptides were employed. Nine patients with advanced prostate cancer androgen were included in the study and no toxic side effects were reported. The transgenic B-lymphocyte immunization induced a T cell response against hTERT in four patients. Moreover, a vaccination strategy against telomerase using hTERT-transfected DCs in 18 patients with metastatic prostate cancer has been tested (Su et al., Proc. ASCO 2004, Abstract 2507). In all subjects, hTERT-specific CTLs could be observed and a biological response (PSA velocity) was shown in a few patients. Finally, Vonderheide et al. [229] recruited seven HLA-A2 positive patients with metastatic breast or prostate cancer for vaccination with DCs pulsed with a HLA-A2 restricted hTERT peptide. hTERT-specific CTLs was induced in four patients and one clinical partial response was associated with induction of CD8+ tumour infiltrating lymphocytes at the site of tumour.

All these data provide a scientific rationale for continued clinical investigations of telomerase immunotherapy strategies. Clearly, immunotherapy against telomerase can induce a few minor clinical responses without major treatment-related side effects. Actually, several American groups have initiated new phase I trials, as the National Cancer Institute which conducts two trials studying the effectiveness of hTERT vaccination in patients with advanced breast, sarcoma or brain cancer. The proof for clinical benefit or not of an hTERT-specific immunotherapy should therefore be established in a near future.

## 5. Gene therapy

All genes have a promoter whose role is to control the expression level. Different tumour-or tissue specific promoters, such as the alpha-fetoprotein promoter in hepatocellular cancer [230], the DF3/MUC1 antigen promoter in breast cancer [231], the prostate-specific antigen promoter in prostate cancer [232–234] and the hTERT promoter [235,236] have successfully been studied in animal models. The approach, however, is limited to specific tumour types that express the corresponding tumour-specific antigens. Therefore, hTERT is an excellent candidate because its expression is reported in about 85% of human primary cancers.

Three different strategies of antitumour gene therapy under the control of tumour/tissue-specific promoters have been developed: (i) induce the expression of proapoptotic genes, (ii) control the assembly of oncolytic viruses, and (iii) activate prodrugs in tumour cells.

Firstly, many studies have used the hTERT promoter to drive proapoptotic genes in vitro. The most significant ones are Bax [237,238], caspases 6–8 [239–241], FAS-associated protein with death domain (FADD) [242,243], and TRAIL [244–246]. A selective expression of these transgenes in telomerase-positive tumours has been achieved.

A second approach has been to consider gene-viral strategies as an oncolytic therapy. Here, the most difficult obstacle has been to achieve viral replication exclusively restricted to tumour cells. Different approaches that have described a tumour-selective viral replication are: (i) introducing deletions of functional gene regions that are essential for efficient replication in normal cells but expendable in tumour cells; (ii) introducing tumour/tissue-specific promoters into viruses to limit the expression of replication genes in normal cells; (iii) changing the viral coat to selectively boost the uptake into tumour cells. Several types of these conditionally replicative (CR) viruses have actually been tested both in preclinical and clinical trials, but a CR adenovirus (CRAD) is the most frequently used [247–250]. It has been shown that the hTERT promoter could be used to regulate tumour-specific expression of genes necessary for viral replication, so that viral replication only occurs in telomerase-positive cancer cells. For example, Su et al. [251] showed that the CRAD virus CNHK300 is excellent in terms of selective replication or oncolytic effects, and is exempt of side effects. On the basis of this knowledge, Zhang et al. [235] used the adenovirus CNHK300-mE, which uses the hTERT promoter to drive the adenoviral *E1A* gene and a mouse endostatin (mE) gene, which is an angiogenic inhibitor. The study was

conducted in a gastric cell line xenograft murine model, monitoring the oncolytic activity and endostatin secretion. The reported results were impressive because only telomerase-positive cancer cells were infected with CNHK300-mE and the virus induced a selective replication of the adenovirus and production of endostatin.

Finally, the "suicide gene" is the third strategy for hTERT-driven cancer gene therapy. Several studies have developed different suicide genes capable of activating prodrugs into cytotoxic drugs in malignant cells. The prodrug induces selective killing of the cancer cells in presence of a suicide gene such as (i) bacterial cytosine deaminase with the prodrug 5-fluorocytosine [252], (ii) the thymidine kinase and ganciclovir treatment [253,254], and (iii) the bacterial nitroreductase for the pro-alkylating agent CB1954 [255,256]. The studies show that gene or viral therapy strategies using the hTERT promoter are potentially safe and show powerful cytotoxic effects. It seems therefore reasonable to propose such strategies in future clinical trials, unless the regulatory authorities remain unwilling to accept trials based on gene therapy.

## 6. Therapeutic potential and limitations

It has long been argued that several criteria are needed to properly validate candidate drugs against telomeres/telomerase. Such criteria usually state that (i) the addition of inhibitors should lead to progressive telomere shortening, (ii) growth arrest/cell death should start after a significant lag-phase, and (iii) the time necessary to observe growth arrest should vary depending on initial telomere length. Effectively, it is known that telomere shortening requires a certain number of cell divisions to become phenotypically manifest. In some models, after targeting telomerase, up to 40 cell doublings have been needed to observe apoptosis or senescence in vitro [93]. Delay in efficacy is therefore an important consideration with agents that target telomerase, in particular in tumours exhibiting long telomeres and/or long doubling times. Thus, initial debulking chemotherapy has been proposed, immediately followed by a prolonged administration of telomerase inhibitors targeting a minimal residual tumour volume [257]. Target the telomerase in the adjuvant setting, or use it in combination with radiation therapy or hormonal therapy to boost tumour response are other potential areas of development. To our knowledge, no experiments have yet explored the potential of radio-sensitisation by hTERT inhibitors. Screening for patients whose tumours are characterized by relatively short telomeres could also be of some interest. Finally, concomitant therapy with telomerase inhibitors and anti-angiogenic agents has also been advocated [258].

However, in recent years, the therapeutic potential of targeting the telomeres or telomerase has been modified by additional arguments. Several of the studies reviewed here, have shown evidence for rapid cell death independently of initial telomere length, and some suggest that telomerase activity may contribute to tumourigenesis independently of its telomere lengthening effects, such as via an anti-apoptotic effect of hTERT [259–262]. It has also been shown that telomerase expression, independently of telomere length, produces resistance to antiproliferative signals of TGFB in cultured human mammary cells lacking p16INK4a [263]. On the other hand, it is believed that apoptosis is achieved by a critical telomere shortening or a telomere structure alteration. There are many recent arguments supporting that changes in the telomere structure itself might induce cell death, also independently of initial telomere length. Indeed, G4 molecules [189–191,193–200], telomere mutation induction by hTR-modification [143], and deprotection of telomeres (as for instance by a dominant negative TRF2) [33] are some examples aiming to take advantage of changes in the telomere structure to induce rapid cell death. As, we have seen in this review, direct telomere targeting is an interesting approach for further drug-development. However, the main problem with direct telomere targeting remains the potential toxicity in telomerase-negative somatic cells. Additionally, inhibition of telomerase could increase tumour malignancy by increasing the genomic instability of the cell. Therefore, rapid, but tumor-specific induced apoptosis should be prioritised. How to achieve rapid apoptosis by targeting telomeres or even telomerase is still needed to be fully explored, but combining several strategies exposed in this review, such as developing vector systems with for instance a dominant negative TRF2 under an hTERT promoter control, could be one of many interesting leads to follow.

Blocking resistance to telomerase inhibitors is another important consideration. The major challenge is probably how to circumvent the ability of tumour cells to solve excessive telomere shortening by mechanisms that do not involve telomerase. The mass of evidence for an alternative mechanism of maintenance of telomere length in human tumours and tumour-derived cell lines is compelling [264]. Therefore, at least theoretically, the use of telomerase inhibitors could increase the risk of selection for ALT-cells. The concept of ALT-related therapy resistance was recently sustained by a study where inhibition of telomerase was achieved in a colon cancer cell line having pre-existing mismatch repair (MMR) defects [265]. The authors used the dominant-negative hTERT approach to suppress telomerase activity and observed the appearance of a telomerase-independent, ALT-like telomere elongation in this specific cell line.

Ultimately, what would be the best-fitted strategy in heterogeneous tumours? Recent work suggests that ALT and telomerase based mechanisms can coexist artificially in some tumour cell lines [266–268] and presumably, human polyclonal tumours might contain cells with either ALT or non-ALT (hTERT+) phenotypes. It means that some human tumours could develop resistance to strategies that target only one of the mechanisms of telomere maintenance and therefore, a combination of both telomerase and ALT inhibitors should be used. However, it has recently been possible to suppress the ALT-mechanism in immortalized fibroblasts and

osteosarcoma cells by overexpressing Sp100, a constituent of promyelocytic leukemia nuclear bodies [269]. The Sp100 protein was in fact sequestering the MRE11, RAD50, and NBS1 recombination proteins away from APBs, resulting in progressive telomere shortening. Therefore, characterization of every molecular component of the ALT mechanism is important to identify new ALT inhibitors as additional targets for drug development. Additionally, as both telomerase and ALT must gain access to the telomere to act, one might expect that at least some of the proteins involved at least partially overlap. So it might be possible to identify common targets for simultaneous inhibition of both mechanisms.

Concerning the predicted toxic side effects of telomerase drugs, one can expect these to be restricted to highly proliferating tissues. Therefore, haematological toxicity is the most expected side effects together with immunologic, cutaneous, and gonadal toxicity. Yet, no impairment of stem cell function has been observed in murine models of anti-telomerase immunotherapy, but mouse and human telomere biology differ in many ways. Therefore, long-term effect on stem cells after telomerase therapy remains unknown. However, there might be specific ways to attenuate toxic side effects of targeting telomerase. For instance, a pre-clinical in vivo study that assessed the interactions between telomere dysfunction and p53 in cells and organs of telomerase-deficient knock-out mice has concluded that the deletion of p53 can significantly attenuate the adverse cellular and organismal effects of telomere dysfunction [98]. Other concerns of general toxicity are related to risks from anti-telomerase approaches using transfection of plasmids and genetic therapy, as for instance when proposing dominant negative experiments. Generally, gene therapy is not yet commonly declared as a risk-free strategy, and might delay, at least clinically, some of the drug developments mentioned in this review. However, future preclinical and clinical studies using telomeres or telomerase as targets for anticancer drug development, will eventually give answers to most of these concerns.

## 7. Conclusive remarks and perspectives

Molecular targeted agents in current clinical development in the cancer arena, include those targeting the self-sufficiency in growth signal (signal transduction inhibitors), the insensitivity to antigrowth signals (cell-cycle modulators), avoidance of programmed cell death (apoptosis modulators), sustained angiogenesis (anti-angiogenic factors), and the tissue invasion and metastatic potential of cancer cells (as src and MMP inhibitors). The unlimited replicative potential is the only hallmark of cancer cells for which targeted agents are yet not available in the clinic. There is however at the preclinical level a multitude of strategies targeting telomerase or telomeres. The very first phase I trials have been implemented, notably in immunology. Nevertheless, additional phase I trials with chemical compounds and early phase II clinical trials are greatly awaited. A way to improve the chances for success of the upcoming clinical trials would be to enrich the study population with mainly selected patients. These would for instance be patients with hTERT positive tumours presenting short telomeres. Selection for such patients would implicate validated biological methods of immunohistochemistry, quantitative fluorescence in situ hybridization (Q-FISH, Flow-FISH), Southern blotting, or other methods such as a recently developed hybridometric assay estimating mean telomere lengths [270]. The choice of candidate telomere/telomerase based drugs to be tested in clinical trials would also depend on further results suggesting a synergistic effect with conventional chemotherapy, or other preclinical results supporting the use of therapeutic cocktails.

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## Biography

*Dr. Jean-Charles Soria* obtained his M.D. degree at the University of Paris-V and carried on with his Ph.D. in fundamental basis of oncogenesis at the University of Paris XI. After a Post-doc training at the MD Anderson Cancer Center in Houston, Texas, US, he practices as a medical oncologist and assistant Professor at the Gustave Roussy Institute, University of Paris XI, since 2001. He is author and co-author of more than 70 scientific publications and is a member of several editorial boards (Lancet Oncology, Journal of Clinical Oncology. . .) and cancer research societies (ESMO, AACR, ASCO, EACR, EORTC. . .).