Targeting telomeres: advances in telomere maintenance mechanism-specific cancer therapies

Jixuan Gao and Hilda A. Pickett ✉

Abstract | Cancer cells establish replicative immortality by activating a telomere-maintenance mechanism (TMM), be it telomerase or the alternative lengthening of telomeres (ALT) pathway. Targeting telomere maintenance represents an intriguing opportunity to treat the vast majority of all cancer types. Whilst telomerase inhibitors have historically been heralded as promising anticancer agents, the reality has been more challenging, and there are currently no therapeutic options for cancer types that use ALT despite their aggressive nature and poor prognosis. In this Review, we discuss the mechanistic differences between telomere maintenance by telomerase and ALT, the current methods used to detect each mechanism, the utility of these tests for clinical diagnosis, and recent developments in the therapeutic strategies being employed to target both telomerase and ALT. We present notable developments in repurposing established therapeutic agents and new avenues that are emerging to target cancer types according to which TMM they employ. These opportunities extend beyond inhibition of telomere maintenance, by finding and exploiting inherent weaknesses in the telomeres themselves to trigger rapid cellular effects that lead to cell death.

Homology-directed repair (HDR). A type of double-strand break repair where a homologous section on a sister chromatid is used as a template to guide DNA synthesis and repair. It involves processing of the double-strand break by the MRN complex to create single-stranded overhangs, prior to RAD51-mediated or RAD52-mediated strand invasion of the sister chromatid to enable DNA extension. Intermediates are then resolved to complete the repair.

Telomere Length Regulation Unit, Children's Medical Research Institute, Faculty of Medicine and Health, University of Sydney, Westmead, NSW, Australia.

✉*e-mail: hpickett@cmri.org.au*

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It has been over 20 years since Hanahan and Weinberg identified replicative immortality as a hallmark of cancer[1] and, in this time, there has been significant progress in the development of therapeutics that alter replicative potential[2,3]. One way to achieve this is through the inhibition of telomere maintenance. Due to the inability of the replication machinery to fully replicate linear DNA molecules, telomeres, which comprise tandem arrays of $(TTAGGG)_n$ repeats at the distal ends of chromosomes, shorten with each cellular division, thereby limiting the lifespan of normal somatic cells[1,4,5]. The double-stranded telomere repeat array terminates in a 100–300-base pair single-stranded guanine (G)-rich 3′ overhang, which loops back on itself, invading proximal repeats of the same telomere, to form a lariat structure known as a telomere-loop (t-loop)[6–9]. Shelterin is a six-subunit protein complex consisting of telomeric repeat-binding factor 1 (TRF1), TRF2, RAP1, TRF1-interacting nuclear factor 2 (TIN2), TPP1 and protection of telomeres protein 1 (POT1) that plays a critical role in stabilizing the t-loop[10–12]. This nucleoprotein structure prevents the chromosome ends from being recognized as DNA double-strand breaks (DSBs) and the inappropriate activation of DNA damage response (DDR) pathways[13,14]. Shelterin binding and formation of the t-loop become compromised as telomeres continue to shorten with repeated cellular divisions, and this diminished protection leads to telomere dysfunction, cellular senescence or apoptosis[13].

The extension of telomeres offers replicative immortality to cancer cells and can be achieved through two major telomere-maintenance mechanisms (TMMs). The first relies on the ribonucleoprotein enzyme telomerase[15,16], and the second involves a homology-directed repair (HDR) pathway referred to as alternative lengthening of telomeres (ALT)[17,18]. While the vast majority of cancer cells have an active TMM, most normal human somatic cells, with the exception of some stem cell populations, lack a TMM. This dichotomy advocates for the manipulation of TMMs as an effective therapeutic strategy for the treatment of most cancer types (BOXES 1 and 2). In this Review, we discuss the opportunities and limitations of targeting TMMs for cancer treatment, with a particular focus on new, repurposed and emerging therapeutics.

Telomerase

The telomerase holoenzyme extends the 3′ ends of linear chromosomes to replenish telomeric repeats that are lost during each round of cell division[19]. Telomerase activity is achieved by human telomerase reverse transcriptase (hTERT), the catalytic component of the enzyme, which

Box 1 | Telomere-maintenance mechanism detection and determination

Telomerase is active in the vast majority of tumour types whilst alternative lengthening of telomeres (ALT) is present in a smaller proportion, with an additional category of tumour types that have no detectable telomere-maintenance mechanism (TMM)[289,290]. The precise proportions of each category are hard to ascertain due to the limited number of reports addressing the prevalence of TMMs, variability in the samples studied, the selected cohort and inconsistencies in the TMM detection technique employed. Nevertheless, it is clear that tumours of mesenchymal or neuroepithelial origin have the highest prevalence of ALT activity, with ALT detected in over 50% of some bone and soft tissue sarcomas[280]. It is unclear why some tumour types favour one TMM over another, although a high prevalence of ALT may reflect tighter suppression of telomerase in the cell type of origin[195]. ALT-positive tumours are biologically and clinically distinct from their telomerase-positive counterparts[291] and, as TMM-specific therapeutics emerge, the need for specific clinical prognostic or diagnostic tests is becoming increasing relevant. Characterization of TMMs in clinical samples has the potential to stratify patients that will benefit from therapeutics targeting each TMM but could also be used retrospectively to determine treatment efficacy in tumours using different TMMs.

Telomerase activity is biochemically measurable whereas a variety of phenotypic markers are used to identify ALT[50,83,292–295]. The most robust and clinically applicable assays for ALT detection are native telomere-fluorescence in situ hybridization (FISH) (known as ALT-FISH), which measures all single-stranded telomeric repeat species in a one-step FISH method, and the C-circle assay[292,294,295]. The C-circle assay involves amplification of extrachromosomal telomeric C-circles using Phi29 polymerase in a rolling circle amplification reaction. Amplification products are then detected using a sequence-specific radiolabelled probe or by quantitative PCR[195]. Significant efforts have also focused on using whole-genome sequencing data to identify the molecular signatures that underlie each TMM in tumours[35,39,40,50]. ALT-positive tumours frequently display loss-of-function mutations in α-thalassemia/mental retardation syndrome X-linked (*ATRX*) or death domain-associated protein 6 (*DAXX*) while telomerase-positive tumours often acquire telomerase reverse transcriptase (*TERT*) modifications such as promoter mutations, amplifications and structural variations[39]. However, the prevalence of such mutations is too low to be prescriptive, loss-of-function mutations in *ATRX* can occur synonymously with hTERT promoter activating mutations, and these mutations are heterogeneously distributed across tumour types[40,296]. Interestingly, the position and prevalence of telomere variant repeats within telomere reads extracted from whole-genome sequencing datasets can effectively determine TMM status[39,40,50]. Telomere variant repeats are telomeric sequences that differ from the canonical telomeric sequence and include TTGGGG, TCAGGG, TGAGGG and TAAGGG. The strength of using genomic signatures to stratify TMMs in tumours is dependent on extensive experimentally validated training and testing datasets, and accuracy can be increased by considering a single tumour type in favour of a tumour-agnostic approach[40]. Currently, TMMs are not considered in cancer diagnosis and prognosis, and these assays require further clinical development and validation.

reverse transcribes telomeric repeats directly onto the chromosome ends, and hTR, an RNA molecule in which the intrinsic telomere template region is embedded. The H/ACA ribonucleoprotein binding factors dyskerin, NOP10, GAR1 and NHP2 as well as the Cajal body chaperone, telomerase Cajal body protein 1 (TCAB1), bind to the core enzyme to facilitate enzyme assembly and confer telomerase complex stability[20–22]. The telomerase essential N-terminal (TEN) domain of hTERT interacts directly with the shelterin component TPP1 to enable the enzyme to bind to the telomeres during S to late G2 phase[23–28]. The interactions between telomerase and telomeres can be both transient and stable, indicative of initial probing associations that precede longer productive interactions[29–33]. Telomere repeat extension commences when the telomeric single-stranded overhang binds to the complementary RNA template (5′-CUAACCCUAAC-3′) in hTR[23–28]. The telomerase active site then moves along the template until it reaches the 5′ boundary, at which point telomerase either dissociates or translocates along the DNA product[23–28] (FIG. 1). Translocation enables processive repeat addition. Extension is not directly coupled to DNA replication and 5′ resection and typically occurs after telomere replication prior to C-strand fill-in[34].

Telomerase is active in germline and most stem cell populations but is repressed in differentiated somatic cells through the silencing of hTERT expression[35–37]. Tight repression of hTERT is facilitated by its heterochromatic genomic environment[38], which is necessary as only a few molecules of hTERT are sufficient to maintain telomere length[39–42]. Cancer cells use a variety of means to re-express hTERT and thereby reactivate telomerase, with hTERT promoter mutations (−124C>T and −146C>T) that can override native hTERT silencing by recruiting the ETS family of transcription factors being amongst the most common pan-cancer driver point mutations[43].

Alternative lengthening of telomeres

ALT is a telomerase-independent TMM that relies on HDR to lengthen telomeres. Hence, ALT-positive cells are defined by their ability to maintain their telomeres in the absence of telomerase. Activation of the ALT mechanism involves the transition of telomeres to a recombination permissive state, which involves altered telomeric chromatin and elevated levels of replication stress (FIG. 2). Loss of either α-thalassemia/mental retardation syndrome X-linked (ATRX) or death domain-associated protein 6 (DAXX) triggers chromatin decompaction, which is thought to be required for the induction of ALT activity[44–49] (FIG. 2a). However, the low prevalence of ATRX and/or DAXX mutations in ALT-positive cancers suggests that chromatin decompaction may aid but is not a necessity for ALT induction[35,50]. Other studies have shown that increases in chromatin compaction by the nucleosome remodelling and histone deacetylase (NuRD)–zinc finger protein 827 (ZNF827) complex and the histone-lysine N-methyltransferase SETDB1 can also stimulate and propagate ALT activity[51,52] (FIG. 2b). Together, these data support the requirement for distinct telomeric chromatin environments for ALT initiation versus ALT propagation and maintenance.

Replication stress can occur when lesions in DNA hinder progression of the replication fork, resulting in stalled forks[53] (FIG. 3). Extensive terminal repetitive sequences and their propensity to form secondary structures, such as G-quadruplexes (G4s), RNA–DNA hybrids, t-loops and displacement-loops (D-loops), make telomeres inherently difficult to replicate[54,55]. The telomeres in cells that use the ALT pathway (ALT telomeres) display exacerbated levels of DNA damage and replication stress due to aberrant telomeric chromatin and altered protein binding[56–59]. The replication stress response protein SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A-like protein 1 (SMARCAL1), along with Fanconi anaemia proteins Fanconi anaemia complementation group M (FANCM) and FANCD2, play vital roles in managing the levels of replication stress at ALT telomeres[60–65] (FIG. 3). These proteins remodel the replication fork, triggering fork regression and re-initiation of replication[61–68]. If unrepaired, stalled forks can deteriorate into DSBs[60–63,65],

Rolling circle amplification reaction
An isothermal DNA or RNA amplification reaction where circular oligonucleotides (for example, C-circles) function as a template for the DNA or RNA polymerase.

5′ Resection
A process where the blunt end of a double-strand break undergoes nucleolytic degradation in the 5′ to 3′ direction to leave a 3′ single-stranded overhang.

Telomere replication
Replication of the telomere repeat tracks.

C-strand fill-in
Telomeres consist of G-strand (5′-TTAGGG-3′) and complementary C-strand (3′-AATCCC-5′) repeats. Telomerase extends the G-strand, resulting in a G-rich single-stranded 3′ overhang. C-strand fill-in is the process by which the complementary C-strand is synthesized by DNA polymerase α-primase 12 to convert the single-stranded DNA of the 3′ overhang into double-stranded DNA.

ultimately promoting the recruitment of DNA repair factors and the engagement of HDR mechanisms that extend the telomeres[69] (FIG. 4). Specifically, 5′-3′ resection of DSBs is achieved by Bloom syndrome helicase (BLM) through its interactions with exonucleases EXO1 and DNA2 (REFS.[70,71]) (FIG. 4a,b). The resulting single-stranded telomeric DNA is then coated with replication protein A (RPA) prior to undergoing homology-directed searches and strand invasion to form a D-loop (FIG. 4a,b).

Several repair pathways are simultaneously engaged at ALT telomeres, with distinct requirements, temporal dynamics and outcomes[72–76]. The predominant telomere extension pathway is dependent on RAD52, with a RAD52-independent pathway operating in situations when RAD52 is compromised[72] (FIG. 4c–e). Both pathways rely on the BLM–DNA topoisomerase 3α (TOP3A)–RecQ-mediated genome instability protein 1 (RMI1) and RMI2 (BTR) complex and its interaction with the proliferating cell nuclear antigen (PCNA)–replication factor C (RFC)–DNA polymerase-δ (Polδ) replisome to mediate branch migration of the D-loop[72,77] (FIG. 4f). The D-loop is then either dissolved by the BTR complex, resulting in telomeric extension without crossover, or resolved via the SLX1–SLX4, MUS81–EME1 and XPF–ERCC1 (SMX) endonuclease complex resulting in crossover events in the absence of telomere lengthening[78–80] (FIG. 4g).

The ALT pathway generates several biomarkers that can be used to identify ALT activity in preclinical studies. As a result of extensive homologous recombination (HR), ALT-positive cells display heterogeneous telomere lengths and increased numbers of telomere sister chromatid exchange events[18]. Telomere synthesis in ALT-positive cells predominantly occurs in a subset of promyelocytic leukaemia (PML) nuclear bodies, known as ALT-associated promyelocytic leukaemia bodies (APBs) (FIG. 4e). APBs are nuclear foci formed by liquid–liquid phase separation that facilitate telomere associations and clustering. APBs are unique to cells using ALT and are comprised of a PML and Sp100 protein shell as well as shelterin components, proteins involved in DNA replication and repair, and telomeric DNA, held together by non-covalent small ubiquitin-like modifier (SUMO)–single-interacting motif (SIM) interactions[81,82]. ALT activity involves break-induced replication (FIG. 4c) and can generate abundant extrachromosomal telomeric repeats (ECTRs), including partially single-stranded, circular structures of C-rich telomeric DNA known as C-circles[83,84]. At least two of these four major ALT markers (heterogeneous telomere lengths, telomere sister chromatid exchanges, APBs and ECTRs) should be present for cells to be classified as ALT positive[17]. Additional parameters can be used in the clinical setting to identify patient tumours as ALT positive and these are described in BOX 1.

Strategies to target telomerase

Therapies that target telomerase have historically been viewed as a highly attractive means of cancer treatment by restraining proliferative capacity through telomere length[85–87] (TABLE 1). However, limitations include the lag phase that ensues following telomerase inhibition as the telomeres shorten to critical lengths before any cytotoxic effects can be observed as well as potential toxicity towards highly proliferative tissue compartments such as haematopoietic and epidermal stem cells that rely on telomerase for regeneration[88,89]. More recently, telomerase-targeting therapies that exert activities beyond inhibiting telomere extension alone have been explored.

Oligonucleotides against hTR. Telomere synthesis can be blocked by oligonucleotides that disrupt hTR function[90] (FIG. 1). The most commonly tested oligonucleotides are N3′ → P5′ thio-phosphoramidates that form stable duplexes with complementary DNA[91]. Cell-permeable 2′-*O*-methyl and N3′ → P5′ thio-phosphoramidate-substituted RNA and/or DNA antisense oligonucleotides

Box 2 | Models for preclinical testing of telomere-maintenance mechanism-specific therapies

A wealth of potential telomere-maintenance mechanism (TMM)-specific protein targets and therapeutic opportunities exist; however, the success of drug discovery for cancers of any type or subtype is dependent on the availability of biologically representative models in which to assess the efficacy of novel agents.

Cell line models

Numerous cell line models exist that have been defined and characterized as having either telomerase or alternative lengthening of telomeres (ALT) activity using a variety of established experimental techniques. These cell lines encompass standard cancer cell lines, patient-derived cell lines and cell lines derived from spontaneously, virally or chemically immortalized fibroblasts. The majority of patient-derived ALT-positive cell lines originate from osteosarcomas, rhabdomyosarcomas, chondrosarcomas and neuroblastomas, consistent with the high prevalence of ALT in these cancer types. Alternatively, isogenic telomerase-positive or ALT-positive cell lines derived from the same genetic background can be utilized to examine TMM-specific effects[297]. An extensive panel of ALT-positive and telomerase-positive clonal cell line derivatives has been established by SV40-immortalization of JFCF-6 mortal jejunal fibroblasts from a male patient with cystic fibrosis[297,298]. Isogenic cell lines have also been derived from SV40-immortalized normal lung IMR-90 fibroblasts and include IMRB (ALT positive) and SW39 (telomerase positive)[299,300], and cellular hybrids of IMRB and SW39 have been established that use either ALT or telomerase[44]. Another relevant cell system is the double-positive GM847 human telomerase reverse transcriptase (hTERT) cell line, which was created by overexpression of hTERT in the ALT-positive GM847 fibroblast cell line and hence displays both TMMs[301]. Cell-based efficacy studies will benefit from examination across an extensive and diverse panel of telomerase-positive and ALT-positive cell lines and mortal cell strains.

Animal models

The majority of animal models used to assess the efficacy of telomerase-targeting therapeutics involve xenografts of telomerase-positive cell lines into immunocompromised mice or rats[302–306]. Syngeneic models have been used to assess the effects of telomerase inhibition on metastatic spread[307]; however, these models are less relevant due to differences between murine and human telomere biology[35,308]. There are fewer animal models for in vivo testing of ALT-targeting therapeutics due to the lack of therapeutic agents and a general lack of assessment of ALT activity. Patient-derived xenografts, established from patients with relapsed neuroblastoma that utilizes either ALT or telomerase, have been used to demonstrate the efficacy of ataxia telangiectasia mutated (ATM) inhibitors in reversing resistance to the commonly used salvage therapy for neuroblastoma, which consists of the chemotherapy combination temozolomide and irinotecan[226]. Patient-derived xenograft models from ALT and/or telomerase-positive sarcoma cell lines have also been established, including the ALT-positive LB857 myxoid sarcoma cell line, which was found to readily form macroscopic tumours in immunocompromised mice[292]. Several ALT-positive osteosarcoma cell lines, such as CAL-72 and SaOS-2, have shown potent tumorigenicity in nude or NOD/SCID-γ-immunodeficient mice[309,310]. Many of these cell lines have been validated for how well they represent osteosarcomas in patients as shown by their ability to produce osteoid. Further development and expansion of the available preclinical models with defined TMMs will be integral to the progress and success of telomerase-targeted and ALT-targeted therapeutics for cancer.

G-quadruplexes
(G4s). Non-canonical secondary structures formed by guanine (G)-rich DNA sequences.

Displacement-loops
(D-loops). D-loops form when single-stranded DNA invades a section of double-stranded DNA, causing it to separate into a loop structure.

are designed to target the template region of hTR[91–94]. When bound, the catalysis of telomere repeat addition can be effectively inhibited. While these oligonucleotides effectively induce telomere shortening, the observed effects are limited to in vitro experiments. The addition of lipid groups can enhance the cell permeability and bioavailability of such oligonucleotides. GRN163L (imetelstat) is a 13-mer N3′ → P5′ thio-phosphoramidate antisense oligonucleotide conjugated to a 5′-palmitoyl group that competitively binds to the hTR sequence template to inhibit telomerase activity[95,96]. Imetelstat has shown in vitro and in vivo efficacy across varying tumour models and remains the only oligonucleotide that has progressed to clinical trials[97–101]. Imetelstat has demonstrated clinical efficacy in myelofibrosis and lower risk myelodysplastic syndromes[99,102–105]. In 2019, after phase II trials, imetelstat was given fast-track designation by the FDA for relapsed or refractory myelofibrosis, a disease

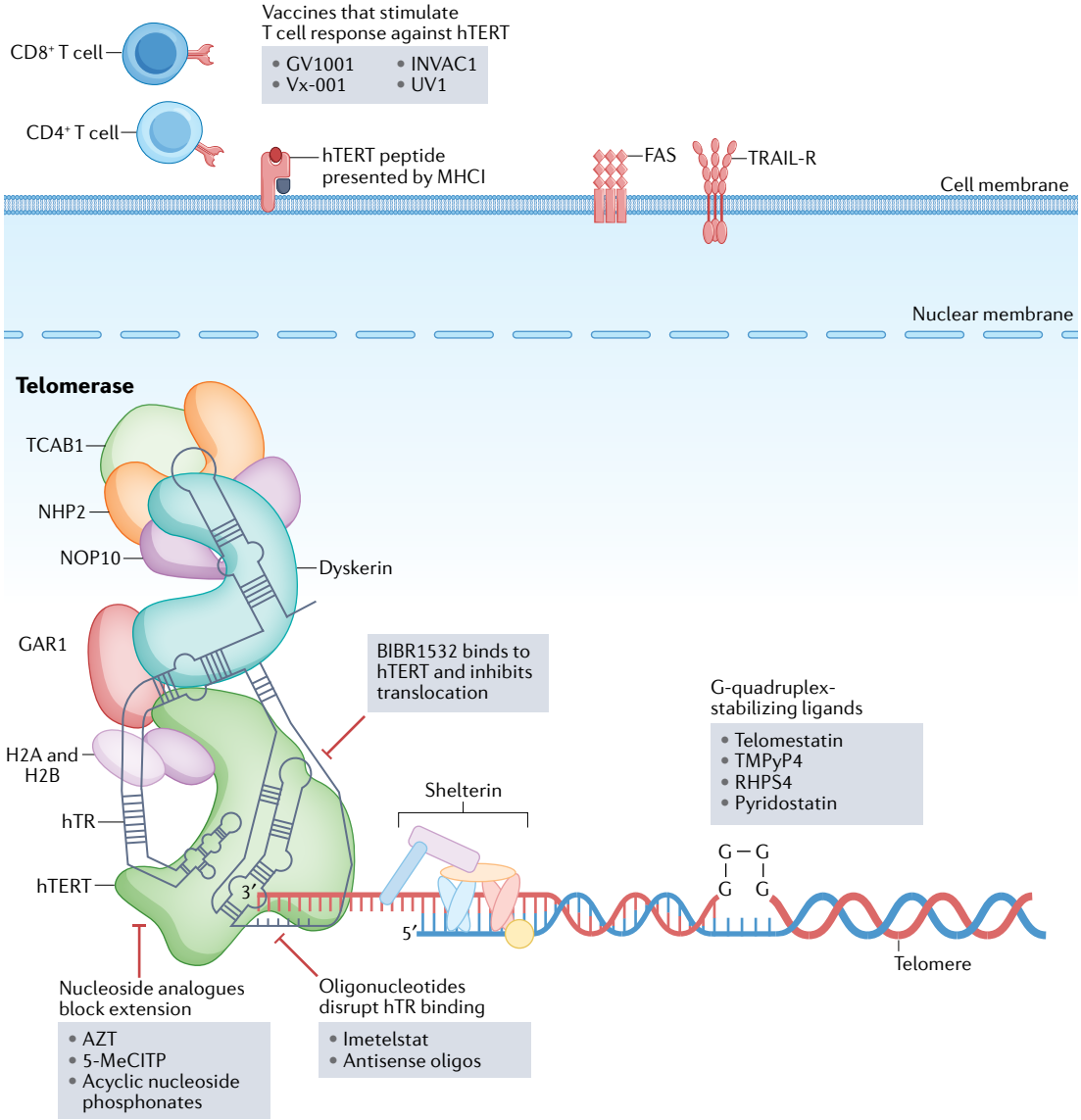


Fig. 1 | **Telomerase-mediated telomere lengthening and the therapeutics that inhibit this process.** Telomerase consists of the human telomerase reverse transcriptase (hTERT) enzyme bound by H/ACA ribonucleoprotein binding factors dyskerin, NOP10, GAR1 and NHP2, the Cajal body chaperone, telomerase Cajal body protein 1 (TCAB1), and hTR, an RNA molecule in which the intrinsic telomere template region is embedded. hTERT peptide vaccines can stimulate immune responses to hTERT peptides presented on the surface of cancer cells. These responses include the release of cytokines or interactions with apoptosis-inducing receptors on cancer cells. The telomeric single-stranded overhang binds to the complementary RNA template (5′-CUAACCCUAAC-3′) in hTR to enable telomeric repeat extension. This interaction can be inhibited by oligonucleotides complementary to hTR. Once telomerase reaches the 5′ boundary, it either dissociates or translocates along the DNA product to enable processive repeat addition. This extension process can be inhibited by BIBR1532, a non-competitive inhibitor of hTERT, nucleoside analogues and G-quadruplex-stabilizing ligands. Illustration of telomerase holoenzyme based on findings from REFS.[15,288]. 5-MeCITP, 5-methylcarboxyl-indolyl-2′-deoxyriboside 5′-triphosphate; AZT, azidothymidine; MHC I, major histocompatibility complex I; TRAIL-R, tumour necrosis factor-related apoptosis-inducing ligand receptor.

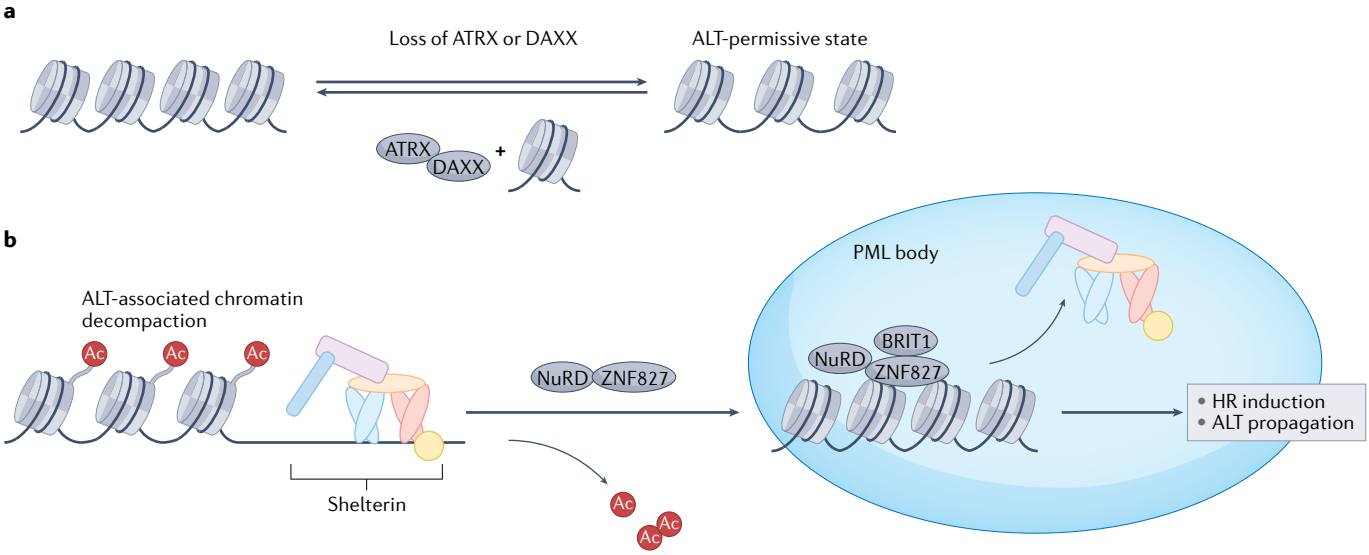


Fig. 2 | **Chromatin remodelling creates an ALT permissive state. a** | The α-thalassemia/mental retardation syndrome X-linked (ATRX)–death domain-associated protein 6 (DAXX) complex forms a histone chaperone complex that deposits histone H3.3 and remodels H3.3-containing nucleosomes at heterochromatic regions, including telomeres. Loss of ATRX and/or DAXX leads to chromatin decompaction, which is thought to promote alternative lengthening of telomeres (ALT). **b** | Nucleosome remodelling and histone deacetylase (NuRD)–zinc finger protein 827 (ZNF827) binding to telomeres using ALT counteracts chromatin decompaction, which contributes to loss of shelterin binding, homologous recombination (HR) and propagation of ALT. PML, promyelocytic leukaemia. Ac, acetylation.

where activated telomerase is thought to maintain the elevated mitotic activity of myelodysplastic cells[106–108].

Despite promising preclinical results in cancer models and efficacy in patients with myelofibrosis, inhibitory effects of imetelstat on progression of other cancer types in humans remain to be seen. Imetelstat has been tested in phase I and II clinical trials on patients with recurrent or refractory solid tumours but only achieved a partial response in a small number of patients[100,103,109]. This response was associated with severe neutropenia and thrombocytopenia, with the latter also leading to intratumoural haemorrhage[100,103,109]. The disparity between the preclinical and clinical results of imetelstat treatment can be explained by the time taken for telomeres to shorten to critical lengths, during which patients can succumb to cancer progression. This lag phase has been demonstrated in vitro in lung cancer cell lines with longer telomeres but these cell lines were often not selected for subsequent in vivo studies, meaning that the response to the agent in cancer xenografts with longer telomeres remains unclear[99]. Data from the clinic supported this notion as patients with shorter telomeres responded better to imetelstat than those with longer telomeres[110]. Due to its severe side effects, continual administration of imetelstat is not possible, creating breaks in the treatment regimen that provide the opportunity for telomere lengths to be restored[99,100,103,109]. Nevertheless, imetelstat remains an efficacious therapeutic for patients with myelofibrosis, and the search for more specific and potent inhibitors of telomerase continues.

Nucleoside analogues. Nucleoside analogues are covalently incorporated at the telomere end by telomerase but prevent further nucleotide addition due to the absence of the 3′-OH functional group (FIG. 1). Due to the structural similarity, numerous analogues that have been previously used to inhibit the human immunodeficiency virus (HIV) reverse transcriptase have also been found to inhibit the hTERT catalytic site[111–113]. Nucleoside analogues that have been investigated in telomerase-positive cancers include azidothymidine (AZT; which is FDA approved for the treatment of HIV), 6-thio-2′-deoxyguanosine (6-thio-dG) and 5-methylcarboxyl-indolyl-2′-deoxyriboside 5′-triphosphate (5-MeCITP)[90,114,115].

Upon entry into cells, AZT is phosphorylated to AZT-triphosphate, which incorporates into DNA to block transcription elongation[114]. AZT was first discovered to cause progressive telomere shortening and growth arrest in the single-celled ciliate *Tetrahymena* and then in human lymphocytes[116–118]. In cancer, the effects of AZT on telomere shortening and cell death have mainly been observed in malignancies induced by viruses such as human T cell leukaemia virus type I (HTLV1)-induced adult T cell leukaemia, acquired immune deficiency syndrome-related Kaposi sarcoma and Epstein–Barr virus-associated lymphoma[119–121]. In the clinic, patients with HTLV1-induced leukaemia treated with AZT showed cancer regression that corresponded with decreased telomerase activity[119]. However, in non-virally induced cancer types, the inhibitory concentration 50 (IC50) of AZT is high (~200–500 μM) and there is little difference in response between cancer types that rely on telomerase versus those that use ALT, suggesting that the observed toxicity may stem from off-target effects[122,123]. 6-Thio-dG serves as a competitive inhibitor of telomerase; it inhibits the growth of both A549 lung cancer and diffuse intrinsic pontine glioma xenografts by inducing both telomere shortening and telomere dysfunction, and has further been demonstrated to cross the

Strand invasion
Single-stranded DNA invades a section of double-stranded DNA with sequence homology.

Osteoid
An unmineralized organic tissue that becomes calcified and contributes to the bone matrix.

Replisome
A protein complex that can exhibit helicase, primase and DNA polymerase activities to replicate DNA of both the leading and lagging strand. During ALT, the replisome consists of proliferating cell nuclear antigen (PCNA), replication factor C (RFC) and DNA polymerase δ (Polδ).

Branch migration
A process that occurs after strand invasion, where one strand of DNA is processively exchanged for another at Holliday junctions or D-loops, resulting in movement of the junction.

Homologous recombination (HR). The most common form of HDR, whereby exchange of genetic material occurs between two homologous chromosomes.

blood–brain barrier, making it an attractive therapeutic for brain tumours[124,125]. Treatment with 6-thio-dG causes telomere uncapping because of failure of the telomere to form the t-loop structure; this causes activation of the DDR, which leads to cell cycle arrest and apoptosis, enabling 6-thio-dG to elicit more rapid cytotoxicity compared to agents that induce cytotoxicity through telomere shortening alone[125]. Although the underlying mechanism of telomere dysfunction by 6-thio-dG is not fully understood, such findings support the development of telomerase-targeting therapies that combine the inhibition of telomere extension with additional activities to achieve enhanced efficacy. Nucleoside analogues can also exert inhibitory effects on telomerase without incorporation into telomeric DNA. 5-MeCITP is an indole nucleotide analogue containing a methoxy group that remains trapped in the catalytic site of telomerase to prevent further telomere extension by telomerase[90]. Unlike AZT, which possesses inhibitory effects on polymerases other than telomerase that heighten its toxicity, 5-MeCITP has fewer off-target effects[90]. Therefore, while 5-MeCITP shows selective toxicity towards telomerase-positive cancer cell lines, it is not as potent as AZT[90].

Small molecule inhibitors of hTERT. The most successful small-molecule inhibitor of hTERT is

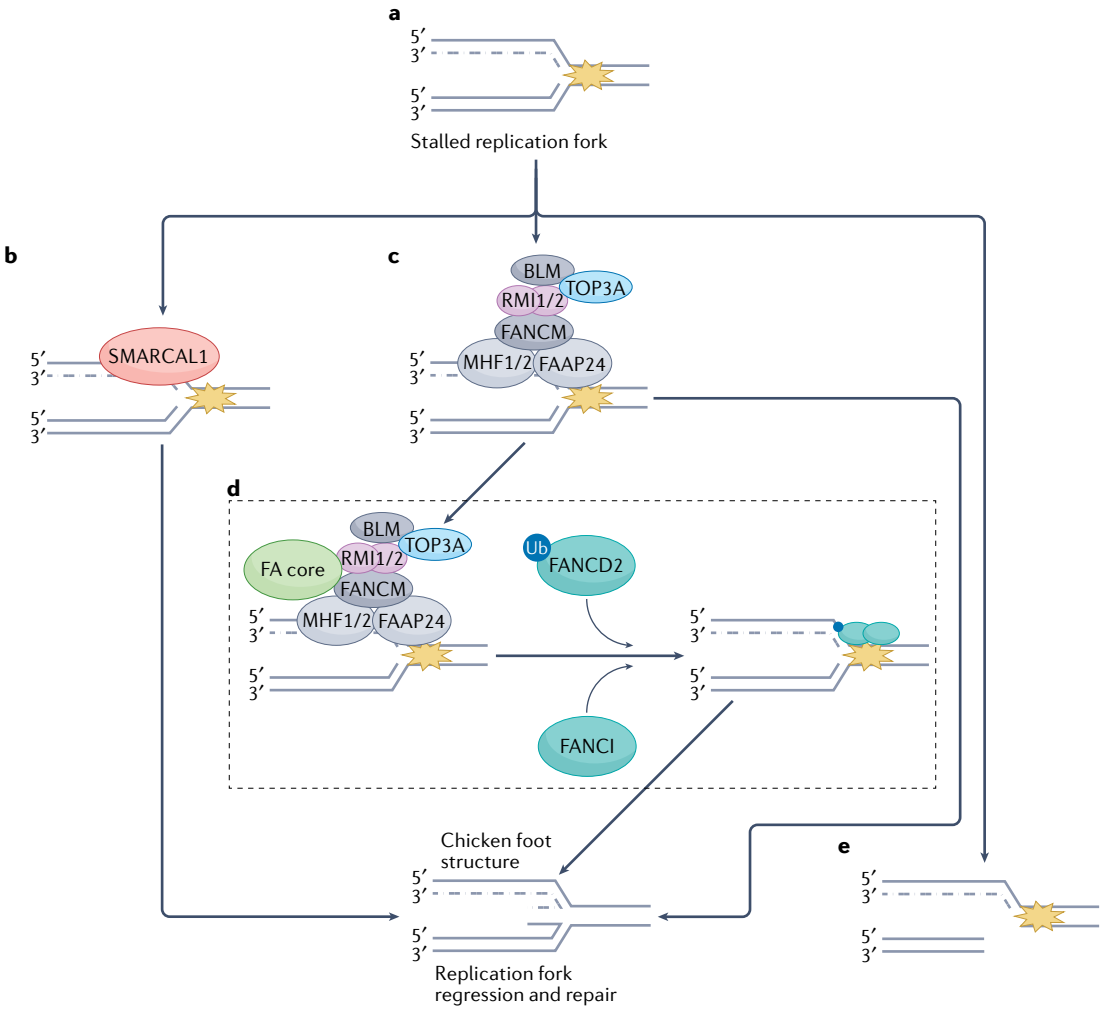


Fig. 3 | **Resolution of replication stress at telomeres using ALT. a** | Complex secondary structures at telomeres using alternative lengthening of telomeres (ALT) make replication difficult, resulting in stalled replication forks. **b** | SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A-like protein 1 (SMARCAL1) is an ATP-dependent DNA annealing helicase that remodels stalled replication forks into four-way chicken foot structures during fork regression to bypass lesions on the parental strand. **c** | Fanconi anaemia complementation group M (FANCM) is a DNA translocase that binds DNA via Fanconi anaemia core complex-associated protein 24 (FAAP24) and MHF1 (also known as CENPS) and MHF2 (also known as CENPX). FANCM forms a complex with Bloom syndrome helicase (BLM), topoisomerase 3α (TOP3A), and RecQ-mediated genome instability protein 1 (RMI1) and RMI2 (known as the BTR complex) to alleviate replication stress by promoting replication fork regression and R-loop resolution. **d** | FANCM independently interacts with the Fanconi anaemia (FA) core complex to monoubiquitinate FANCD2, which complexes with FANCI and binds to sites of DNA damage to induce fork regression. **e** | If left unrepaired, stalled replication forks deteriorate into double-strand breaks. The star represents a block on the DNA template that prevents or slows the progression of the replication machinery. These blocks can be secondary structures such as G-quadruplexes, 8-oxo-G (8-oxo-guanine) lesions, t-loops or D-loops.

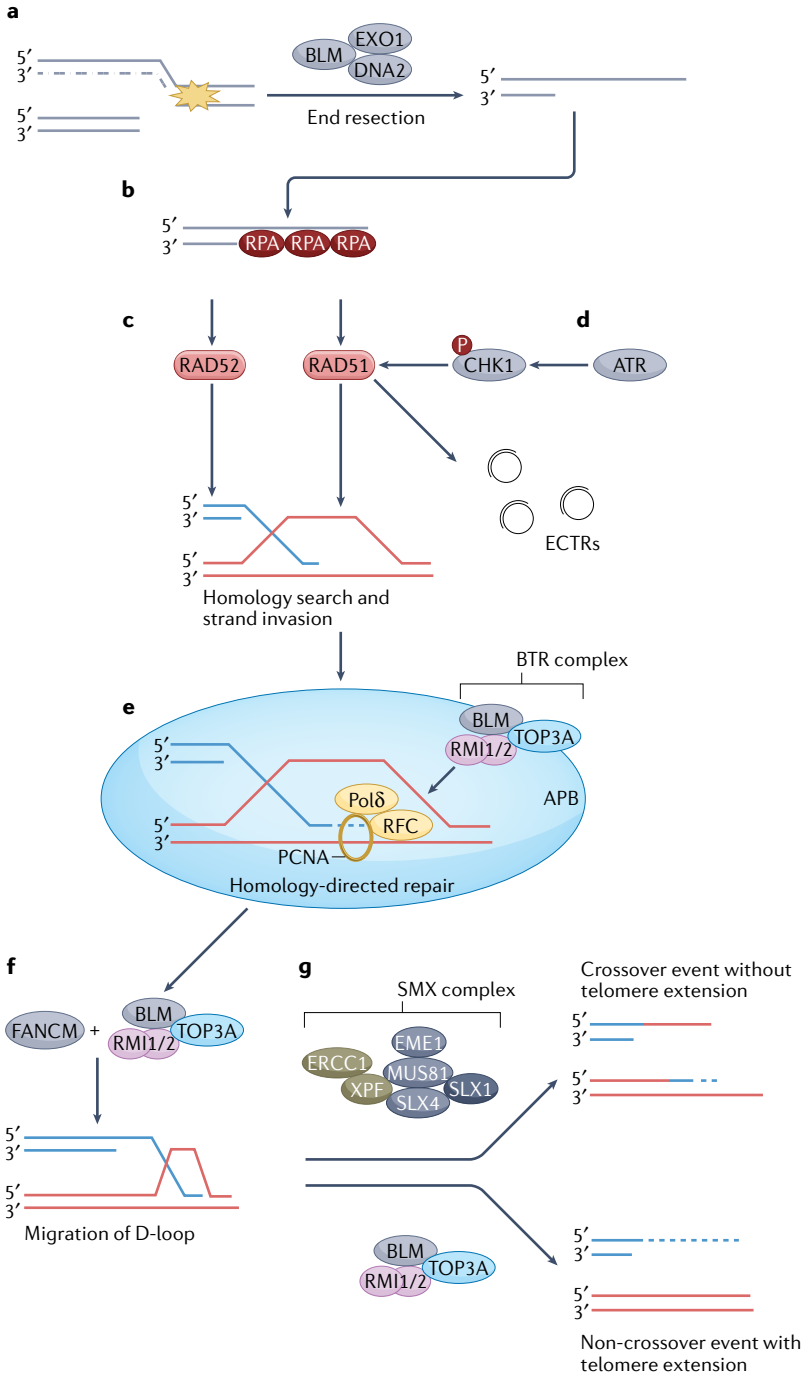


Fig. 4 | **Multiple DNA repair pathways are engaged at telomeres using ALT. a** | Bloom syndrome helicase (BLM), through its interactions with exonucleases EXO1 and DNA2, performs 5′-3′ resection of double-strand breaks. **b** | The resulting single-stranded telomeric DNA is then coated with replication protein A (RPA) prior to undergoing homology-directed searches and strand invasion to form a D-loop. **c** | Several repair pathways are simultaneously engaged at telomeres using alternative lengthening of telomeres (ALT), with distinct requirements, temporal dynamics and outcomes. The predominant extension pathway is dependent on RAD52, with a RAD52-independent pathway operating in situations where RAD52 is compromised. **d** | ATM and RAD3-related (ATR)–checkpoint kinase 1 (CHK1) signalling stabilizes and restarts replication forks whilst inhibiting cell cycle progression until all lesions are repaired. Inhibitors of ATR disrupt this replication stress response to mediate apoptosis. **e** | Both RAD51-mediated and RAD52-mediated homologous recombination pathways rely on BLM, topoisomerase 3α (TOP3A), and RecQ-mediated genome instability protein 1 (RMI1) and RMI2 (the BTR complex) and its interaction with the proliferating cell nuclear antigen (PCNA)–replication factor C (RFC)–DNA polymerase-δ (Polδ) replisome to mediate telomere extension. RAD51-dependent recombination generates extrachromosomal telomeric repeats (ECTRs). Telomere repeat DNA synthesis occurs in ALT-associated promyelocytic leukaemia bodies (APBs). **f** | During telomere extension, the BTR complex together with Fanconi anaemia complementation group M (FANCM) enable branch migration of the D-loop. **g** | The D-loop is then either dissolved by the BTR complex, resulting in telomeric extension without crossover, or resolved via the SLX1–SLX4, MUS81–EME1, XPF–ERCC1 (SMX) endonuclease complex, resulting in crossover events in the absence of telomere lengthening.

2-[(E)-3-naphthalen-2-yl-but-2-enoylamino]-benzoic acid (BIBR1532)[126–129]. BIBR1532 was discovered from an in vitro screen and found to be selective for telomerase[126]. BIBR1532 binds to hTERT at a non-catalytic site, thereby inhibiting telomerase activity with non-competitive kinetics[130] (FIG. 1). Generally, non-competitive inhibitors are more potent as they do not have to compete with endogenous substrates. BIBR1532 does not block the initial telomere extension event but instead interacts with the hydrophobic pocket of the thumb domain of telomerase to prevent the further translocation required for processive telomere repeat addition[126,130,131]. Despite the inhibitory effects of BIBR1532 on telomere extension in cancer cells, the cytotoxic effects of BIBR1532 appear to arise from direct damage to telomere structure. Specifically, BIBR1532 treatment resulted in loss of TRF2 binding, which induced markers of telomere dysfunction, including telomere end-to-end fusions and increased activation of p53 (REF.[132]). In preclinical studies, BIBR1532 has demonstrated potent effects in a number of cancer cell lines and xenograft models, including breast cancers, fibrosarcomas, endometrial cancers and leukaemias[126,133–138]. BIBR1532 also has the propensity to sensitize resistant cancer cells to chemotherapy, which has important clinical implications as chemoresistance is one of the main causes of cancer progression and mortality[139]. Sensitization appeared to be correlated to telomere shortening as prolonged treatment with BIBR1532 heightened sensitivity; however, the precise mechanism is unknown.

Another method to inhibit hTERT via small-molecule inhibitors is to block transcription of the *hTERT* gene[140]. Inhibition of *hTERT* transcription can be achieved through the green tea polyphenol epigallocatechin gallate (EGCG), which inhibits DNA methyltransferase 1 (DNMT1)[141,142]. This causes the *hTERT* promoter to become hypomethylated, allowing binding of the Rb–E2F1–histone deacetylase 1 (HDAC1) repressor and causing suppression of *hTERT* transcription[142]. Since EGCG is relatively unstable, compounds synthesized with EGCG-related moieties have been explored as improved hTERT inhibitors. Of these compounds, chemical screens have identified MST-312 as a potent

ALT-associated promyelocytic leukaemia bodies (APBs). Membraneless structures formed by phase separation that promote the aggregation of homologous recombination proteins, nucleases, telomere-associated proteins, PML proteins and telomeric DNA. APBs are a biomarker of alternative lengthening of telomeres (ALT).

Table 1 | **Major telomerase-targeting therapeutics in preclinical and clinical development**

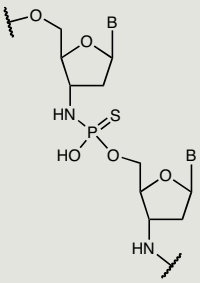
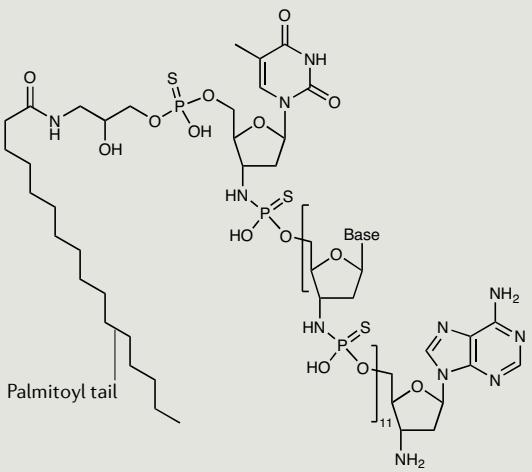
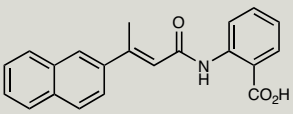
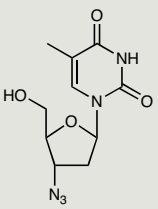
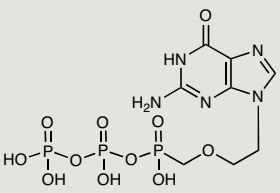
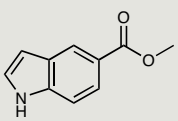
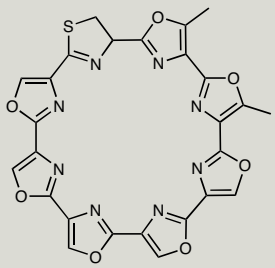
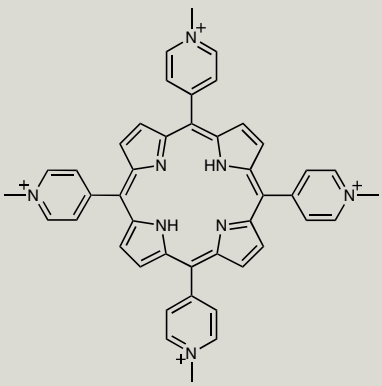
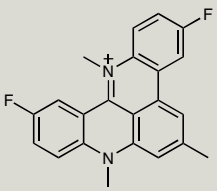
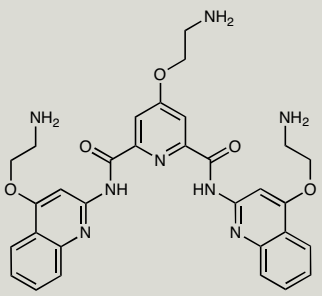
Target	Compound	Mechanism	Stage of development	Structure of compound (if available)
hTR	N3′→P5′ thio-phosphoramidate	Binds to the template region of hTR to prevent catalysis of telomere repeat addition[93]	Preclinical	
	2′-*O*-methyl-RNA	Binds to the template region of hTR to prevent catalysis of telomere repeat addition	Preclinical	NA
	Imetelstat	Lipid-conjugated phosphorothioate 13-mer antisense oligonucleotide that binds to hTR to inhibit telomerase[95]	FDA approved for myelofibrosis	
hTERT	BIBR1532	Binds to non-catalytic site on hTERT to inhibit translocation[130]	Preclinical	
Telomeres (nucleoside analogues)	AZT	Covalently incorporated into telomeres but the absence of a 3′-OH group prevents further nucleotide addition[111]	FDA approved for HIV	
	Acyclic nucleoside phosphonates, for example, PMEGpp	Covalently incorporated into telomeres but their absence of 3′-OH group prevents further nucleotide addition[281,282]	Varies with individual inhibitor, for example, PMEA (adefovir) is FDA approved for hepatitis B, but the most potent inhibitor of telomerase, PMEGpp, is still at preclinical stages	
	5-MeCITP	Traps itself in the catalytic site to inhibit telomere extension[90]	Preclinical	

Table 1 (cont.) | **Major telomerase-targeting therapeutics in preclinical and clinical development**

Target	Compound	Mechanism	Stage of development	Structure of compound (if available)
$CD8^+$ T cells	GV1001	hTERT peptide-containing vaccines that stimulate a $CD8^+$ T cell response against hTERT-presenting cancer cells	Phase III clinical trials (NCT02854072)[283]	NA
	Vx-001	hTERT peptide-containing vaccines that stimulate a $CD8^+$ T cell response against hTERT-presenting cancer cells	Phase II clinical trials (NCT01935154)[284]	NA
G-quadruplex stabilizers	Telomestatin	Stabilizes G-quadruplexes to prevent telomere extension and shelterin binding, leading to replication stress[178]	Preclinical	
	TMPyP4	Stabilizes G-quadruplexes to prevent telomere extension and shelterin binding, leading to replication stress[285]	Preclinical	
	RHPS4	Stabilizes G-quadruplexes to prevent telomere extension and shelterin binding, leading to replication stress[286]	Preclinical	
	Pyridostatin	Stabilizes G-quadruplexes to prevent telomere extension and shelterin binding, leading to replication stress[287]	Preclinical	

5-MeCITP, 5-methylcarboxyl-indolyl-2′-deoxyriboside 5′-triphosphate; AZT, azidothymidine; HIV, human immunodeficiency virus; hTERT, human telomerase reverse transcriptase; NA, not available; PMEA, 9-(2-phosphonomethoxyethyl)adenine; PMEGpp, 9-(2-phosphonylmethoxyethyl)guanine diphosphate.

inhibitor of hTERT[140]. Treatment of cells of various cancer types, including breast, lung and colon carcinomas, with MST-312 caused downregulation of hTERT expression, decreased telomerase activity, and telomere shortening, which led to cell cycle arrest and apoptosis[142–145]. However, cytotoxic effects took up to

Small ubiquitin-like modifier
(SUMO). Units that are covalently attached to proteins post-translationally in a process known as sumoylation. This can alter several properties of the protein, including protein stability, localization, and addition or removal of protein–protein binding sites.

Break-induced replication
Recombination-dependent DNA synthesis that initiates from a double-strand break and occurs following strand invasion mediated by RAD51 or RAD52.

Extrachromosomal telomeric repeats
(ECTRs). Linear and circular extrachromosomal copies of telomeric sequences that are generated during homologous recombination in cells using ALT, including C-circles and t-circles. ECTRs are a biomarker of ALT.

Myelofibrosis
A rare type of bone marrow cancer that prevents the production of blood cells, leading to anaemia and scar tissue in the bone marrow.

Transcription elongation
A step in RNA transcription that occurs following initiation and prior to termination when the RNA sequence is synthesized complementary to the DNA template.

Inhibitory concentration 50
(IC50). The dose of an agent required to inhibit 50% of cell growth.

Epitope spreading
The process by which epitopes, distinct from the inducing epitope of a vaccine, become major targets of the immune response.

Macrocyclic compound
A compound made up of chemical ring structures that each consist of 12 or more carbon atoms.

Porphyrin
A molecule that consists of a ring of four linked heterocyclic groups that can be held together by a central metal atom.

90 days post-treatment to occur due to the time required for telomeres to shorten to critical lengths[140]. During this lag phase, cells can also adapt to the loss of telomerase activity, as shown when cells returned to their original growth rate after prolonged treatment (>200 days)[146]. For tumours with relatively short telomeres, MST-312 does exert anti-oncogenic effects in vivo[145]. In vitro MST-312 treatment also appears to result in off-target effects that may enable it to exert more rapid cytotoxicity, including inhibitory effects on topoisomerase II and nuclear factor-κB (NF-κB)[144,145,147]. This may account for the synergism observed between MST-312 and the chemotherapeutic drug doxorubicin, which is observed during short-term experiments[147].

MST-312 and BIBR1532 remain the most promising small-molecule inhibitors of hTERT. Nevertheless, a range of screens have identified other inhibitors. These include Nu-1 and rubromycin antibiotics, which directly inhibit the catalytic domain of hTERT, and bufalin and rapamycin, which block *hTERT* transcription[148–152]. These agents have all stalled at early stages of preclinical studies mainly due to low potency and slow onset of cytotoxic effects. Evaluating telomerase inhibitors based on their ability to induce telomere dysfunction rather than telomere shortening per se may provide a more accurate indicator of potency against telomerase-positive cancer cells.

Immunotherapies against hTERT. Telomerase can be therapeutically targeted through vaccines that stimulate an immune response against surface hTERT[153]. Cancer cells can process endogenous hTERT and present hTERT peptides on the cell surface through major histocompatibility complex (MHC) I and II molecules[154] (FIG. 1). hTERT vaccines typically comprise peptides of the enzyme, which are injected into the dermis where dendritic cells present the antigens to CD4$^+$ T helper 1 (T_H1) cells in the lymph nodes[154,155]. These hTERT-specific T_H1 cells migrate into the tumours, where they either stimulate CD8$^+$ T cell activity against hTERT-expressing cancer cells, or kill the cancer cells directly by releasing cytokines or interacting with FAS or tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) apoptosis-inducing receptors[154,155].

GV1001 is an early generation vaccine based on a 16-amino acid peptide that spans the hTERT active site and has been shown to significantly extend the survival rates of patients with pancreatic cancer that had a CD8$^+$ T cell response[156]. However, as with many immunotherapies, a number of studies have shown that a substantial proportion (around 40%) of patients did not show an immune response to GV1001 (REFS.[156–160]). Combining GV1001 with chemotherapy to enhance the immune response has been explored but with limited success[160,161]. Specifically, GV1001 combined with temozolomide resulted in an immune response in 78% of patients with stage IV melanoma, resulting in longer survival rates in responders compared with non-responders although the difference was not statistically significant (median overall survival 396 days versus 250 days)[161]. Combination treatments of GV1001 with the chemotherapeutic drugs capecitabin or gemcitabine failed to prolong survival beyond chemotherapy alone[160]. Given the less-than-optimal results of these phase III trials, the focus of research into telomerase vaccines has shifted to other hTERT vaccines, including UV1, Vx-001 and INVAC1.

The UV1 vaccine was developed against the epitopes generated from epitope spreading events following treatment with GV1001 (REF.[162]). UV1 stimulates a wide range of CD4$^+$ and CD8$^+$ T cell responses against hTERT[163]. When administered in combination with the cytokine granulocyte–macrophage colony-stimulating factor (GM-CSF) to patients with late-stage prostate cancer, the vast majority of patients had stable disease for the 9 months of the study, exceeding observations with GV1001 (REF.[164]). UV1 is currently in phase II clinical trials (NCT04382664)[165] and the FDA has now granted fast-track approval of UV1 in combination with anti-PD1 therapies for the treatment of advanced malignant melanoma. The Vx-001 vaccine, which contains two hTERT peptides, has shown long-lasting immunogenicity and survival extension in patients with various types of cancer[166–168]. Long-lasting immune responses were observed in around 30% of patients with non-small-cell lung carcinoma and these responders, including those who would have had poor prognosis, had significantly longer survival rates than non-responders (21.3 and 13.4 months, respectively; $P = 0.004$)[166]. However, given the low response rates, no extension in survival was observed when comparing the overall cohort of vaccinated versus unvaccinated patients[166,168].

INVAC1 is a DNA-based vaccine that consists of a plasmid encoding an inactivated form of hTERT fused to ubiquitin[153,169]. When expressed, hTERT is degraded by the proteasome, allowing its antigens to be presented to stimulate an immune response[153,169]. In mouse sarcoma models, INVAC1 stimulated a broad range of hTERT-specific immune responses, including the generation of high numbers of CD4$^+$ T_H1 effector and memory CD8$^+$ T cells, which led to a 50% increase in survival rates[169]. Phase I clinical trials of INVAC1 found that the vaccine was well tolerated, triggered hTERT-specific CD4$^+$ and CD8$^+$ T cell responses, and prevented cancer progression in 58% of patients with relapsed or refractory solid tumours[169,170]. INVAC1 has now progressed to phase II clinical trials (NCT03265717)[171].

G4-stabilizing ligands. G4 ligands were initially thought to be negative regulators of telomerase activity by inhibiting telomerase binding to telomeric DNA[172,173]. Consistent with this rationale, unwinding of telomeric G4s by POT1 is necessary for normal telomerase processivity and to prevent telomerase stalling[174–176]. Numerous G4-stabilizing ligands have been developed, including telomestatin, TMPyP4, RHPS4 and pyridostatin, which vary substantially in their ability to inhibit telomerase binding and processivity[177] (FIG. 1). Telomestatin is a naturally occurring macrocyclic compound that prevents telomerase from extending telomeres by inducing the formation and stabilization of intramolecular antiparallel G4s[178]. Telomestatin treatment impairs telomere extension and is associated with cytotoxicity in a range of cancer cell lines and xenografts[179–181]. The porphyrin

Non-homologous end-joining
A repair pathway where double-strand breaks are ligated together. Non-homologous end-joining (NHEJ) consists of either canonical NHEJ or alternative NHEJ. In the canonical pathway, the two ends of the DNA are bound by Ku70 and Ku80 and DNA-PKcs, which come together to form the synaptic complex. This is then ligated together by the ligase IV–XRCC4 complex. Alternative NHEJ occurs independently of canonical NHEJ proteins and involves the direct joining of short sequence homologies (microhomologies).

TMPyP4 facilitates the formation of intermolecular G4, has potent inhibitory effects on telomerase activity and can inhibit tumour growth in vivo[182,183]. However, one of the major caveats of telomerase inhibition by G4 ligands is the prevalence of G-rich DNA with the propensity to form G4s throughout the genome, including in the promoter regions of oncogenes[184]. G4 ligands are therefore likely to confer significant off-target effects. It has also been shown that a subset of telomeric G4 structures that exist in parallel conformations can be extended by telomerase albeit at a lower affinity to that of linear DNA, suggesting that telomerase extension is compatible with the presence of G4 stabilizers[185–187].

Strategies to target ALT

One of the greatest challenges in the search for ALT-selective cancer therapies is that the factors involved in ALT are also critical to normal DNA replication and repair. Therefore, much of the drug discovery process for ALT-positive cancers is centred around the search for druggable targets that, when suppressed, exert selective toxicity to ALT-positive cancers. It has also been observed that ALT activation can serve as a resistance mechanism to prolonged anti-telomerase therapy, meaning that patients with telomerase-positive tumours may require ALT-targeting therapeutics post-relapse[188–191]. It is unclear whether such resistance mechanisms stem from telomerase inhibition causing activation of ALT or from the selective expansion of a subpopulation of cancer cells using ALT, which originate from a heterogeneous tumour cell population with both TMMs. Both options are feasible and should be considered[192–194]. The aggressive nature of ALT-positive cancers combined with the stagnancy of treatment regimens for many of the tumour types with high ALT prevalence indicate that ALT is primed for therapeutic targeting[195–197]. Several therapeutic avenues are currently under investigation.

Repurposing therapies. Poly(ADP-ribose) polymerase 1 (PARP1) is a member of the PARP family of ADP-ribosyl transferases and is one of the earliest responders to DNA damage, acting as a sensor and repair protein for both single-strand breaks and DSBs[198–201]. PARP1 consumes NAD^+ to add poly(ADP-ribose) chains onto itself as well as histones at the sites of damage, which serve as platforms for the recruitment of DNA repair proteins[202]. PARP inhibitors have particular utility in HR-deficient cancers, such as breast and ovarian cancers with BRCA1 or BRCA2 mutations, through the induction of synthetic lethality[203–207]. In these cancer types, PARP inhibitors bind and trap PARP onto DNA, blocking replication fork progression and causing DNA damage. In ALT-positive cancers, the mechanism of action appears to differ. Preclinical studies have shown that, in ALT-positive cancer cells, PARP inhibitors cause TRF2 dissociation from telomeres, thereby stimulating inappropriate non-homologous end-joining repair[208,209]. This resulted in lethal telomere fusions and culminated in apoptosis and impaired growth of ALT-positive intracranial astrocytomas in mice[208,209]. Several PARP inhibitors are now FDA-approved for HR-deficient breast, ovarian, pancreatic and metastatic prostate cancers[210–213]. Based on the preclinical data, testing PARP inhibitors in patients with ALT-positive cancers warrants further investigation[210,214].

Oncolytic herpes simplex virus type 1 (HSV1) can selectively infect, replicate within and induce the lysis of cancer cells[215]. A modified version of this virus, known as Talimogene laherparepvec (T-VEC), is the first-in-class oncolytic HSV1 to have received FDA approval for the treatment of metastatic melanoma[216]. HSV1 has several attractive characteristics from a therapeutic perspective; first, it has the ability to infect a wide range of cancer types; second, it can be treated by antivirals in the event of disease arising from the infection; and third, it can be targeted to cancer cells through modifications to its glycoproteins[217–219]. The rationale for exploring HSV1 as a treatment strategy for ALT-positive cancers stems from the ability of ATRX and DAXX to create intrinsic antiviral resistance to HSV1 infection[220–222]. ALT-positive cancer cells can exhibit loss-of-function ATRX and/or DAXX mutations, suggesting that these cancer types would be more susceptible to HSV1 infections. This was demonstrated using a mutant HSV1 that lacks the ability to degrade PML nuclear body components, which was found to be more effective at infecting ATRX-deficient cancer cells than ATRX-positive cells[223]. Since high numbers of PML nuclear bodies (often observed in ALT-negative cells) confer resistance to HSV1, the inability of the mutant HSV1 to degrade PML nuclear bodies may explain the target cytotoxicity of the virus towards ALT-positive cancer cells[223]. Infection with the mutant HSV1 also selectively reduced the proportion of human osteosarcoma U-2 OS cells, which are ALT positive and lack ATRX, when co-cultured with ALT-negative fibroblasts, indicating selective toxicity to ALT-positive cells[223]. These data support further investigation of HSV1 for the treatment of ALT-positive cancer types with ATRX and/or DAXX mutations.

Replication stress modulators. ALT-positive cells have characteristically high levels of telomere-specific replication stress that perpetuate ALT-mediated HDR pathways[53,60,61,63]. The level of telomeric replication stress in ALT-positive cells is finely balanced to achieve the maintenance of telomere length and cell viability without the cell succumbing to toxic levels of telomere damage[61]. If this balance is tipped to favour either outcome, the result can have profound effects on cell fate. Targeting the proteins involved in resolving replication stress or components of the replication stress response pathway has the potential to exacerbate telomeric DNA damage and offer selective toxicity to ALT-positive cancer types. The major advantage of this approach is the rapid cytotoxic response to telomere stress induction that minimizes adaptive responses and the potential emergence of resistance mechanisms.

The development of inhibitors of ataxia–telangiectasia mutated (ATM) and ATM and RAD3-related (ATR) protein kinases for cancer treatment has established significant momentum in recent years. ATM contributes to the replication stress response in the context of DSBs, suggesting that ATM inhibitors may exacerbate telomere replication stress and cell death in ALT-positive cancer types[224]. Several ATM inhibitors have been developed.

Mitotic DNA synthesis
The process of DNA repair synthesis during mitosis.

Specifically, AZD0156 has shown selective toxicity in ALT-positive neuroblastomas and is currently in phase I clinical trials (NCT02588105)[225,226]. Furthermore, resistance to the chemotherapeutic drugs temozolomide and SN38 (the active metabolite of irinotecan) used upon relapse was reversed when neuroblastoma cell lines were treated with AZD0156 (REF.[226]). ATR is recruited to sites of replication stress to stabilize and restart replication forks and inhibit cell cycle progression until all lesions are repaired[227–230]. There are currently multiple ATR inhibitors at various stages of clinical development, including four inhibitors in phase I (BAY1895344 and VX-803) and phase II (VE822 and AZD6738) clinical trials[231]. ATR inhibitors also sensitize cancer cells to DSB-inducing chemotherapeutic drugs, such as cisplatin and melphalan, because such damage requires ATR-mediated HR repair[232,233]. It was previously shown that ATR inhibitors were more toxic to ALT-positive cancer cells than to telomerase-positive cancer cells[234]. This initial discovery provided optimism for the treatment of ALT-positive cancers; however, the selectivity of ATR inhibitors for ALT-positive cancers has since been called into question[235,236].

The potency of ATR inhibitors can be enhanced by combining them with PARP inhibitors. This has been demonstrated in a variety of cancer models but none have been validated to be ALT positive[237–240]. However, in the context of ALT-positive cancer types, a clinical trial is under way, albeit still in the recruiting stage, to examine the safety and efficacy of the ATR inhibitor M1774 alone and in combination with the PARP inhibitor niraparib (NCT04170153)[241,242]. In this trial, the presence of ALT is determined by the presence of loss-of-function mutations in ATRX and/or DAXX. Other clinical studies are in progress to test novel ATR inhibitors, such as RP-3500 and BAY 1895344, but while activation of the DDR will be examined via histone variant γH2AX staining, assessment of the ALT status of patient tumours and telomere-specific DNA damage will not be conducted (NCT04267939, NCT04497116)[243,244]. The design of these clinical trials highlights a major limitation to the discovery of ALT-based therapeutics, which is that tumour ALT status is often not determined or is predicted using loosely correlative markers such as loss of ATRX and/or DAXX. Assessing ALT activity using more robust tests, such as the C-circle assay, ALT–fluorescence in situ hybridization (FISH), and telomere variant repeat content, has the potential to uncover new and more robust anti-ALT activities of agents currently in clinical trials (BOX 1).

FANCM is a DNA translocase that attenuates replication stress specifically at telomeres using ALT by promoting branch migration, which results in replication fork regression and the displacement of D-loops and R-loops[63,245–247]. FANCM depletion causes cells to enter a hyper-ALT state, characterized by increased telomere damage and a dramatic induction of ECTR DNA, that is ultimately toxic to cells using ALT[61]. Proof-of-principle experiments to disrupt the FANCM–BTR complex have yielded encouraging results in cancer cells using ALT. Specifically, inducible expression of a peptide, corresponding to the MM2 domain of FANCM, that sequesters BTR complex components away from endogenous FANCM, resulted in C-circle induction, telomere damage and a striking loss of viability of cancer cells using ALT[61]. Similarly, ALT-positive cancer cell lines showed increased sensitivity to the small-molecule inhibitor PIP-199, which was identified through a high-throughput small-molecule screen of approximately 75,000 compounds as an inhibitor of the FANCM–BTR protein–protein interaction[61,248]. While PIP-199 appears to show ALT-selective toxicity at sub-micromolar concentrations, this compound requires substantial further validation and optimization.

The propensity for telomeres to form G4s implicates these secondary structures as obstacles for the replication machinery[249]. Consequently, G4-stabilizing ligands have the potential to exacerbate telomeric replication stress in cells using ALT. Telomestatin, previously discussed for its role in inhibiting telomerase-mediated telomere extension, can also destabilize shelterin complex binding, causing telomere replication stress and resulting in mitotic arrest[250]. The pentacyclic acridine compound RHPS4 has been shown to induce phenotypes associated with ALT, including telomere dysfunction, fragility and recombination, as well as causing an increase in APBs and C-circles, all of which can be attributed to increased levels of telomeric replication stress[251]. Pyridostatin and 2,6-pyridine-dicarboxamide derivatives have also been shown to cause an increase in fragile and dysfunctional telomeres and an increase in telomeric mitotic DNA synthesis in cells using ALT[73,252]. One limitation is the observation that replication stress caused by G4-stabilizing ligands was able to fuel recombination and drive ALT activity[251], opposing any therapeutic potential and highlighting the need for potential therapeutics that target replication stress to strike the correct balance between activating ALT-associated HDR pathways and killing ALT-positive cells.

Potential novel therapeutic targets. Characterization of the ALT mechanism has led to the discovery of an increased number of factors involved in the ALT process. Some of these factors are redundant, while the suppression of others can severely impair the mechanism of ALT or trigger the death of cells using ALT. Therefore, target discovery in this case involves delineating between redundant factors and true therapeutic targets as well as assessing the targets for druggability. Although the pathways discussed below do not yet have reliable inhibitors, their importance to cancer types using ALT advocates that they should be considered in the development of future ALT-targeting therapies.

The cyclic GMP-AMP synthase (cGAS)–stimulator of interferon genes (STING) pathway is part of the innate immune system that senses both host and foreign cytosolic double-stranded DNA to initiate a type I interferon response[253,254]. In the context of cancer, the interferon response leads to apoptosis, thus providing a critical tumour-suppressive function[253,254]. Although the exact nature of the self-double-stranded DNAs that trigger cGAS activation is not completely understood, ECTR DNA generated by cells using ALT can stimulate this interferon response. Specifically, fibroblasts exposed

to ECTRs triggered cGAS–STING-dependent DNA sensing, which led to enhanced interferon signalling and impaired cell proliferation[255]. In cancer cells using ALT, where ECTRs are abundant and can translocate into the cytoplasm, the cGAS-STING pathway is typically defective due to low STING expression and, consequently, these cells using ALT exhibit an impaired interferon response[255]. This highlights two major weaknesses of cells using ALT. First, while the impaired interferon response enables cells using ALT to evade ECTR-induced anti-proliferative effects, it may also confer vulnerability to viral infections. Second, the necessity for cells using ALT to shut down the cGAS–STING pathway is indicative of the potent lethality of this pathway to these cells if active. This suggests that testing the end-products of this pathway, such as the FDA-approved interferon-β (IFNβ), may be a rational approach to inhibit the growth of ALT-positive cancer cells[256–262].

Several strategies to disrupt APB formation have been investigated. One method involves targeting the SUMO ligases to disrupt the SUMO–SIM interactions that hold the PML components together. Suppression of the MMS21 SUMO ligase of the structural maintenance of chromosomes protein 5 (SMC5)–SMC6 complex, which prevents sumoylation of the shelterin components TRF1 and TRF2, causes APB disruption, telomere shortening and senescence in cancer cells using ALT[263]. This implicates inhibition of the ligases that mediate sumoylation as a viable therapeutic strategy for cancer types using ALT. Ubiquitin carrier protein 9 (UBC9) is the sole E2-conjugating enzyme in the sumoylation cascade and plays an important role in sumoylation of the PML protein. 2′,3′,4′-Trihydroxy flavone (2-D08) is a small-molecule inhibitor that inhibits the transfer of SUMO from the UBC9 thioester conjugate to SUMO substrates[264] and has the potential to inhibit the sumoylation of PML proteins and the formation of APBs, causing cytotoxic effects in cells using ALT[265]. A major caveat is that 2-D08 is likely to exert widespread inhibition of sumoylation at other UBC9 SUMO substrates in addition to PML proteins[266].

APB formation also enables proximity-dependent degradation of shelterin components by ubiquitin ligases. Specifically, the shelterin component POT1 colocalizes with the ubiquitin-specific-processing protease 7 (USP7) deubiquitinase within APBs. Testis-specific Y-encoded-like protein 5 (TSPYL5) has recently been identified as a PML component and functions as a USP7 inhibitor[267]. Suppression of TSPYL5 activates USP7 (REF.[267]), which allows USP7 to deubiquitinate and stabilize POT1-targeting ubiquitin ligases that would otherwise undergo auto-ubiquitination and degradation[267]. The active POT1-targeting ligases then ubiquitinate POT1, resulting in its proteasomal degradation[267]. Without POT1, the shelterin complex becomes compromised, causing telomere dysfunction and cell death[267]. Interestingly, the interaction between USP7, POT1 and its ubiquitin ligases was dependent on APB formation, explaining why TSPYL5 suppression was specifically toxic to ALT-positive cancer cells[267]. This study not only identifies TSPYL5 as a possible therapeutic target for ALT-positive cancer types but also raises the possibility of targeting interactions and processes that occur uniquely within APBs as a treatment strategy for ALT-positive cancers.

Other proteins within the shelterin complex, specifically TRF1 and TRF2, have also been explored as potential therapeutic targets for cancer treatment. TRF1 and TRF2 form homodimers that bind to double-stranded telomeric DNA[268]. TRF1 can recruit helicases, including BLM and regulator of telomere elongation helicase 1 (RTEL1), to remove secondary structures, thereby preventing replication stress[54,269,270]. TRF1 also suppresses ATR signalling during S-phase, which would otherwise induce a fragile telomere phenotype[54]. The ability of TRF1 loss to elicit cytotoxicity by inducing DNA damage has been investigated using TRF1-knockout mouse models. TRF1 knockout reduces the progression of several cancer types, including glioblastoma and lung cancer, while the knockout has minimal impact on organ function in non-malignant tissues and on survival of mice, suggesting the possibility of a therapeutic window[271–273]. This therapeutic window was demonstrated when tolerable doses of small-molecule inhibitors against TRF1 (ETP-47228 and ETP-47037), which inhibit TRF1 binding to DNA, induced DNA damage and inhibited cancer progression[271,273]. TRF1 function can also be indirectly inhibited through the use of kinase inhibitors due to TRF1 stabilization and foci formation being dependent upon TRF1 phosphorylation by ERK2, BRAF, mTOR and AKT kinases[272,274].

Mutations in TRF2 result in altered telomeric DNA topology that can initiate an ATM-dependent DDR[275]. TRF2 also cooperates with RAP1, another component of the shelterin complex, to suppress the localization of PARP1 and SLX4 to telomeres, thereby inhibiting non-homologous end-joining[275,276]. Reduced recruitment of HDR proteins to telomeres results in telomere resection, telomere loss and chromosome fusions[276]. Triazole-stapled peptides have been developed to block the protein–protein interaction between RAP1 and TRF2, which functions to suppress inappropriate HDR[277]. The interaction between TRF2 and the 5′-exonuclease, Apollo, presents another druggable opportunity. Apollo, when recruited to the telomeres by TRF2, creates the 3′ single-stranded overhang, which invades the proximal regions of the telomere to form the t-loop. Cyclic peptide mimetics of the TRFH-binding motif on Apollo can bind to TRF2, disrupting its interaction with Apollo[278]. The TRFH domain on TRF2 itself has also be inhibited by cyclic peptides, resulting in activation of the DDR[279]. The efficacy of shelterin inhibitors has not been studied in the context of different TMMs. Given the importance of shelterin and t-loops in preventing telomere dysfunction irrespective of the TMM of the cancer, it is possible that inhibitors of shelterin function may exhibit a ‘pan-cancer’ cytotoxic effect.

Conclusions and future perspectives

Telomerase inhibitors have been the focus of substantial interest and investment over the last few decades, while ALT-positive cancers have remained ignored and therapeutically unchallenged. However, the landscape is changing, and ALT is rapidly becoming recognized as a

specific clinical classification, with the potential to be therapeutically targeted. ALT is estimated to be active in 10–15% of all cancer types, with its prevalence reaching >50% in some subtypes of bone and soft tissue sarcomas and central nervous system tumours[280]. As ALT-positive cancer types are typically aggressive and recalcitrant to current treatment regimens, there is an urgent need to diagnose and effectively treat this significant group of cancers.

In this Review, we have discussed the detection techniques amenable for the clinical diagnosis of ALT and collated current progress in the development of disruptors of both telomerase-mediated and ALT-mediated telomere lengthening pathways as cancer therapeutics. While many promising inhibitors have been developed against telomerase, including the highly attractive hTERT vaccines and imetelstat, which gained fast-track FDA approval, recent major developments have focussed on new therapeutic opportunities for patients with ALT-positive cancer types. In line with our growing understanding of the ALT mechanism, inhibitors that fortuitously target components of the ALT pathway, which have been developed over many years, are becoming increasingly recognized and relevant for their utility in the treatment of ALT-positive cancer types. To date, many of the molecular-based treatments for ALT come from repurposing inhibitors developed primarily for other cancer types but novel inhibitors, such as the FANCM inhibitor PIP-199, are emerging as targeted and ALT-selective therapeutics. The major challenge now is to comprehensively test these approaches in cell and animal models as well as in the clinic to establish efficacy and TMM-selective toxicity. In summary, the field is poised to recognize and treat cancer types based on their TMM status, with ALT-targeted therapeutics offering a broad-based precision approach for the treatment of a significant proportion of tumour types.

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