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Review

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Author for correspondence:
Quintino Giorgio D'Alessandris,
E-mails: giorgiodal@hotmail.it; quintinogiorgio.dalessandris@policlinicogemelli.it

Telomerase inhibition in malignant gliomas: a systematic review

Quintino Giorgio D'Alessandris[1], Marco Battistelli[1,2], Giovanni Pennisi[1,2], Martina Offi[1,2], Maurizio Martini[3], Tonia Cenci[3], Maria Laura Falchetti[4], Liverana Lauretti[1,2], Alessandro Olivi[1,2], Roberto Pallini[1,2,*] and Nicola Montano[1,2,*]

[1]Department of Neurosurgery, Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy; [2]Department of Neuroscience, Neurosurgery Section, Università Cattolica del Sacro Cuore, Rome, Italy; [3]Department of Pathology, Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy and [4]CNR-Institute of Biochemistry and Cell Biology (IBBC), Monterotondo, Rome, Italy

Abstract

Glioblastoma (GBM) is the most frequent adult malignant brain tumour and despite different therapeutic efforts, the median overall survival still ranges from 14 to 18 months. Thus, new therapeutic strategies are urgently needed. However, the identification of cancer-specific targets is particularly challenging in GBM, due to the high heterogeneity of this tumour in terms of histopathological, molecular, genetic and epigenetic features. Telomerase reactivation is a hallmark of malignant glioma. An activating mutation of the hTERT gene, encoding for the active subunit of telomerase, is one of the molecular criteria to establish a diagnosis of GBM, IDH-wildtype, in the 2021 WHO classification of central nervous system tumours. Telomerase inhibition therefore represents, at least theoretically, a promising strategy for GBM therapy: pharmacological compounds, as well as direct gene expression modulation therapies, have been successfully employed in *in vitro* and *in vivo* settings. Unfortunately, the clinical applications of telomerase inhibition in GBM are currently scarce. The aim of the present systematic review is to provide an up-to-date report on the studies investigating telomerase inhibition as a therapeutic strategy for malignant glioma in order to foster the future translational and clinical research on this topic.

Introduction

Glioblastoma (GBM), *IDH*-wildtype, is the most frequent adult brain malignant tumour. The current standard of care combines surgery, radiotherapy (RT) and temozolomide chemotherapy; nevertheless, the median overall survival has not substantially improved over the last years and still ranges from 14 to 18 months (Ref. 1). Thus, novel therapies are urgently needed. Identification of cancer-specific targets is particularly challenging in GBM, as these tumours are highly heterogeneous in terms of histopathological, molecular, genetic and epigenetic features (Ref. 1). Accordingly, targeted therapies have generally failed to improve GBM prognosis, except for some experiences with personalised treatments (Ref. 2). In 2021, the World Health Organization updated the classification of central nervous system tumours (Ref. 3). In the novel scheme, GBM, IDH-wildtype, has been clearly separated from IDH-mutant tumours, including astrocytoma, IDH-mutant, grade 4 (formerly known as GBM, IDH-mutant).

Telomerase reactivation represents one of the key mechanisms leading to tumourigenesis in malignant glioma in general (Ref. 4). Telomerase is a ribonucleoprotein complex built up by multiple subunits (Ref. 5). The core catalytic subunit is the telomerase reverse transcriptase (hTERT), which is tightly associated to a non-coding template telomerase RNA (hTR or hTERT). hTERT-hTR complex is bound to other proteins, including histones H2A and H2B and H/ACA ribonucleoproteinase. Telomerase, in turn, is associated with shelterin, another protein complex which mediates the interaction between telomerase and telomeres. Telomerase allows maintaining telomere length, thus leading to cell immortalisation. Conversely, telomerase knockdown leads to cell senescence, DNA damage and finally to apoptosis (Ref. 6). One of the key mechanisms leading to telomerase upregulation in GBM, IDH-wildtype, is an activating mutation of the hTERT gene, encoding for the active catalytic subunit of telomerase. Mutations occur at hotspots C228T or C250T, creating de novo bindings sites for GA binding protein transcription factors (GABPs), thus fostering hTERT mRNA transcription (Ref. 7). Conversely, in IDH-mutant astrocytomas, ATRX mutation induces the telomerase-independent alternative lengthening of telomeres (ALT) machinery (Ref. 3). The percentage of GBM tumours that express telomerase enzyme activity varies between 26 and 67% (Ref. 8). This variability probably reflects the high heterogeneity of GBM. In fact, where sampling of multiple regions from the same tumour were analysed, 100% of GBMs were found to show telomerase activity (TA) (Ref. 8). A high intra-tumoural TA is predictive of poor prognosis (Ref. 4). The strong negative prognostic role of hTERT promoter mutation in GBM, IDH-wildtype, has been incorporated in the 2021 WHO classification, in which this

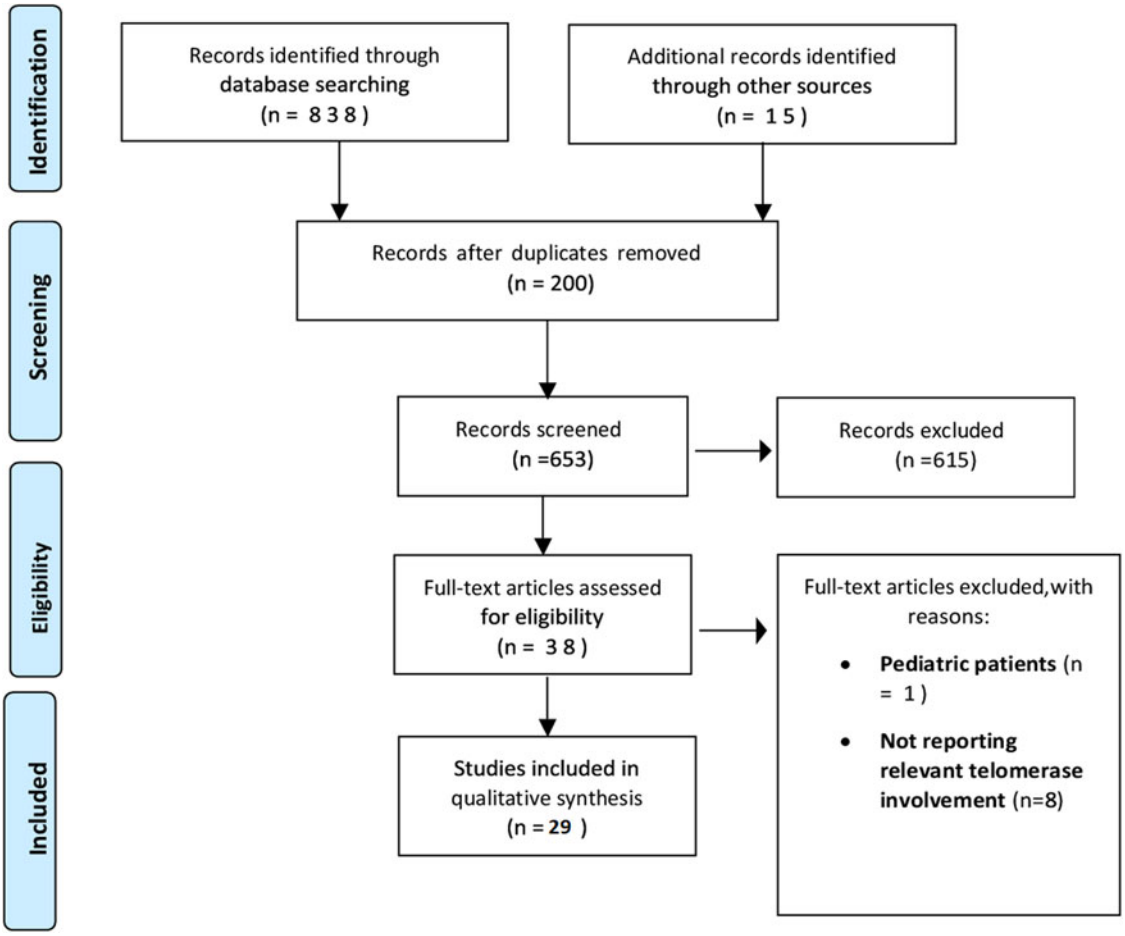

Fig. 1. PRISMA flow diagram.

mutation is sufficient to establish a diagnosis of GBM even in the absence of necrosis and microvascular proliferation (Ref. 3).

Based on this background, therapies aiming at reducing TA are of promising effectiveness in malignant gliomas, particularly in GBM, IDH-wildtype. The aim of this systematic review was to report on available options of telomerase inhibition in order to provide an overview for future developments in this field of research.

Methods

A search in the PubMed and Scopus databases was launched in July 2021 using, as search terms, GBM, Glioblastoma, high grade glioma, TERT and telomerase in any possible combination. PRISMA guidelines were followed (Ref. 9). After duplicate removal, 653 records were screened and 38 full-texts were assessed for eligibility. Of these, nine fell outside the scope of the present review. Twenty-nine papers were included in the final review (Fig. 1).

Results

Pharmacological therapies

Pharmacological therapies that have been exploited to obtain telomerase inhibition are listed in Table 1. In the following paragraphs, we have categorised these treatments based on the main mechanism of action.

Compounds acting through transcriptional hTERT inhibition

Mirzazadeh *et al.* (Ref. 10) studied the effect of *resveratrol* (*RSV*), a polyphenolic compound, over hTERT expression in U87MG cells. hTERT mRNA expression appeared to be significantly decreased in RSV-treated cells as compared to controls, and this resulted in a reduction of cell viability.

Another phytochemical compound, the monoterpene *thymoquinone* (*TQ*), was shown to be able to induce cell death in GBM cell lines, mediated by hTERT inhibition and reduced TA (Ref. 11). TQ was more active on DNA-PKcs knockout lines, like M059J, than on DNA-PKcs wt lines like M059K, pointing at the complex relationship between telomerase and DNA double-strand break repair machinery. Cell death, however, was time-dependent and occurred only several days after the drug was added to the culture.

A research group from Singapore conducted elegant in vitro studies on the effects of biological molecules over GBM cell lines through modulation of telomerase (Refs 12, 13, 14, 15). *Curcumin*, *plumbagin*, *genistein* and *trichostatin A* (*TSA*), a specific histone deacetylase (HDAC) inhibitor, were tested over radioresistant KNS60 and U251MG and radiosensitive GGM A172 GBM cell lines, and over medulloblastoma ONS76 cells. All these drugs determined a reduction in hTERT mRNA levels due to transcription inhibition. Concerning TSA, its HDAC inhibition ability was mechanistically responsible of this effect; however, the exact molecular cascade triggered by the other drugs was less clear. The final mechanism by which all these compounds determined cell viability reduction was cell-cycle arrest due to blockade at G2/M checkpoint (Refs 12, 13, 14, 15). Curcumin, plumbagin and TSA displayed cytotoxic effect on telomerase-expressing brain tumour cells (Refs 12, 13, 14), while genistein was mainly cytostatic (Ref. 15). Differences in the amount of hTERT mRNA and TA reduction were detected

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Table 1. List of pharmacological therapies for telomerase inhibition

Author (year)	Type of study	Drug	Mechanism of action	Conclusions	Clinical level
Mirzazadeh *et al.* (Ref. 10)	*In vitro*	Resveratrol (RSV)	Inhibition of hTERT transcription	Cell growth inhibition. The molecular mechanism for transcriptional hTERT inhibition is unclear	Yes
Gurung *et al.* (Ref. 11)	*In vitro*	Thymoquinone (TQ)	Inhibition of hTERT transcription	Indirect hTERT inhibition linked to DNA-PKc	Yes
Khaw *et al.* (Ref. 12)	*In vitro*	Trichostatin A (TSA)	Inhibition of hTERT transcription due to histone deacetylase inhibition	Exposure to TSA resulted in apoptosis in a dose-dependent manner in the brain cancer cells	Yes
Khaw *et al.* (Ref. 13)	*In vitro*	Curcumin	Inhibition of hTERT transcription	Curcumin-induced growth inhibition and cell-cycle arrest at G2/M phase in GBM and medulloblastoma cells, with consequent induction of apoptosis	Yes
Khaw *et al.* (Ref. 14)	*In vitro*	Plumbagin	Inhibition of hTERT transcription	Plumbagin-induced DNA damage, cell-cycle arrest and apoptosis in GBM and medulloblastoma cells	No
Khaw *et al.* (Ref. 15)	*In vitro*	Genistein	Inhibition of hTERT transcription	Genistein-induced growth arrest by G2/M cell-cycle blockade	Yes
Lin *et al.* (Ref. 16)	*In vitro* and *in vivo*	Butylidenephthalide (BP)	Inhibition of hTERT transcription through Sp1 blockade	BP inhibits proliferation and induces senescence in human GBM	Yes
Kiaris and Schally (Ref. 17)	*In vitro* and *in vivo*	GH-RH antagonist MZ-5-156	Inhibition of hTERT transcription	Preliminary evidence of GHRH antagonists needing expansion and validation	No
Udroiu *et al.* (Ref. 18)	*In vitro*	Epigallocatechingallate (EGCG)	Inhibition of hTERT transcription	EGCG-induced senescence and genotoxicity, probably not only through hTERT inhibition but also exploiting other mechanisms	Yes
Das *et al.* (Ref. 19)	*In vitro*	Retinoids: all-trans-retinoic acid (ATRA), 13-cis-retinoic acid (13.CRA)	Inhibition of TERT transcription	Retinoids-induced differentiation with downregulation of telomerase activity and enhanced the sensitivity to IFN-gamma and TXL (taxol)-induced apoptosis	Yes
Lavanya *et al.* (Ref. 20)	*In vitro*	BIBR1532: 2-[(E)-3-naphtalen-2-yl-but-2-enoylamino]-benzoic acid	hTERT inhibition	Cytotoxic effect on LN18 GBM cell line, mainly inducing apoptosis	No
Biray Avci *et al.* (Ref. 21)	*In vitro*	BIBR1532	hTERT inhibition	Reduced proliferation rate and cytotoxicity in U87 glioblastoma cells, by epigenetic modulation	No
Ahmad *et al.* (Ref. 23)	*In vitro* and *in vivo*	Costunolide	Indirect TERT inhibition	Telomerase inhibition dependent on ROS production and p53 activity	No
Gurung *et al.* (Ref. 24)	*In vitro*	MST-312 (EGCG derivative)	TERT inhibition	MST-312 treatment decreased brain tumour cell viability, induced cell-cycle arrest and double-strand breaks (DSBs). The authors suggest a strategy of double telomerase-DNA-PKc inhibition	No
Takahashi *et al.* (Ref. 25)	*In vitro* and *in vivo*	Eribulin	TERT-RNA-dependent RNA polymerase inhibition	Effective in suppressing GBM xenograft growth. Potential repositioning as GBM treatment	Yes
Marian *et al.* (Ref. 26)	*In vitro* and *in vivo*	Imetelstat (GRN163L)	hTR inhibition	Reduced proliferation and induced death in GBM tumour-initiating cells. Synergistic effect with radiotherapy and temozolomide	Yes

(*Continued*)

Table 1. (*Continued.*)

Author (year)	Type of study	Drug	Mechanism of action	Conclusions	Clinical level
Ferrandon *et al.* (Ref. 27)	*In vivo*	Imetelstat (GRN163L)	hTR inhibition	Significant tumour reduction in mice bearing orthotopic GBM xenograft treated with RT + imetelstat	Yes
Ozawa *et al.* (Ref. 28)	*In vivo*	GRN163	hTR inhibition	Antitumour effect in GBM xenografts	No
Bejarano *et al.* (Ref. 29)	*In vitro and in vivo*	TRF1 inhibitors (ETP-47228, ETP-47037 and ETP-50946) on human GBM cells	TRF1 (sheltering member) inhibitions	TRF1 inhibitors caused DNA damage and reduced stemness of GBM cells, and reduced also xenograft growth	No
Zhou *et al.* (Ref. 30)	*In vitro*	G4 ligand BRACO-19	G-quadruplex telomeric stabilisation	BRACO-19 suppresses proliferation and reduces telomerase activity in human GBM cells, paralleled by the displacement of telomerase from nuclear to cytoplasm	No
Lagah *et al.* (Ref. 31)	*In vitro*	G4 ligand 3,11-difluoro-6,8,13-trimethyl-8H-quino[4,3,2-kl] acridinium methosulfate (RHPS4)	G-quadruplex telomeric stabilisation	Growth inhibition and cell-cycle arrest in tumour cells	No

among the different cell lines and drug tested, and some conflicting results were also detected, in which TA was modulated despite modest changes in hTERT mRNA expression. (Ref. 14).

Lin *et al.* (Ref. 16) assessed the dose-dependent anti-hTERT effect of *butylidenephthalide* (*BP*), the major component of the chloroform extract *Angelica sinensis*. They firstly observed that in vitro BP treatment (25–100 ug/ml) of glioma cells DBTRG-05MG decreased hTERT mRNA and reduced TA by 48 h; this effect was mediated by Sp1 downregulation and p16/p21 upregulation. Reportedly, Sp1 is able to bind to specific sites of hTERT promoter, fostering gene transcription, thus Sp1 blockade is an effective anti-hTERT strategy. The anti-proliferative hTERT-related effects of BP on GBM cells were confirmed in subcutaneous xenografts of DBTRG-05MG GBM cells. To examine the effect of BP on cell proliferation, GBM-derived cells GBM-8401, transfected or not with hTERT, were treated with BP. By immunofluorescence analysis, BP reduced proliferation index and arrested cell cycle only in wild-type GBM cells, but not in hTERT-upregulated ones.

Kiaris and Schally (Ref. 17) tested the activity of a *growth hormone releasing factor antagonist*, *MZ-5-156*, over TA on glioma U87MG cells and other cell lines. The drug was able to significantly decrease hTERT expression and reduce TA activity, both in vitro and in vivo xenografts, in a c-myc-independent manner. However, effects on tumourigenicity and proliferative ability of GBM cells were not demonstrated, nor the exact mechanism of action elucidated.

Udroiu *et al.* tested the effect of chronic *epigallocatechin gallate* (*EGCG*) treatment over radioresistant U251MG cells for 98 days (Ref. 18). EGCG treatment significantly reduced hTERT mRNA, TA and cell growth rate, and induced senescence as assessed by increase in β-galactosidase activity. However, no effects on cell viability were detected.

Das *et al.* investigated in vitro in C6 rat glioma cells the effects of *all-trans retinoic acid* (*ATRA*) and *13-cis retinoic acid* (*13-CRA*) on TA (Ref. 19). Retinoids are widely known to induce cell astrocytic differentiation (Ref. 19). Telomerase repeated amplification protocol (TRAP) assay detected reduced TA, along with reduced rat telomerase reverse transcriptase (rTERT) at RT-PCR and western blot (WB) assay. This resulted in an increased sensitivity to taxol- and IFN-gamma-induced apoptosis.

Compounds acting through direct hTERT inhibition

Lavanya *et al.* (Ref. 20) examined in vitro the effect of *BIBR1532* on GBM cell line LN18. BIBR1532 is a non-peptidic, non-nucleoside small molecule telomerase inhibitor that specifically binds to the active site of hTERT. Administered in a wide dose range (25–200 μM), BIBR1532 showed a dose-dependent ability to reduce cell viability and induce cytotoxicity. Also hTERT mRNA levels appeared to be reduced in a dose-dependent fashion, and WB documented parallel reduction of hTERT protein expression. TA was accordingly reduced. Annexin-V staining elucidated that BIBR1532 growth inhibitory effect was mainly mediated by apoptosis. Considered these results, another research group (Ref. 21) tested the epigenetic effect of BIBR1532 on GBM U87MG cells. BIBR1532 increased apoptotic rate and induced cytotoxicity also in U87MG cell line. In treated cells, an increased expression of epigenetic controlling proteins, such as HDAC and DNA methyltransferase, was found. These results highlight that BIBR1532 is efficient in modifying epigenetic mechanisms involved in telomerase expression in U87MG cells.

Compounds acting through other hTERT inhibition

Costunolide (*CS*) is a sesquiterpene lactone acting as a telomerase inhibitor. In vitro studies conducted on breast cancer cell lines MCF-7 and MDA-MB-231 had demonstrated that CS-induced

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telomerase inhibition exerted an anti-proliferation activity through cell-cycle arrest at G1/S and G2/M checkpoints, mediated by p21 and p53 upregulation (Ref. 22). Moreover, CS was shown to induce cell apoptosis by activating both the intrinsic pathway (through upregulation of p53, Bax and caspase-3, and downregulation of Bcl-2) and the extrinsic pathway (by downregulating NF-κB/p65 complex). Finally, CS caused reactive oxygen species (ROS) accumulation which produced ROS-dependent cell senescence and apoptosis (Ref. 22). In glioma, Ahmad *et al.* demonstrated that CS induced p53-mediated glioma cell death triggered by ROS induction. In fact, only p53-wt glioma cell lines, like A172 and U87MG, were sensitive to CS treatment, while p53-mutated cell lines like T98G were not (Ref. 23). The authors suggest that telomerase inhibition is indirect, mediated through ROS induction and p53 activity. In vivo, CS administration reduced tumour volume and weight in subcutaneous glioma xenografts in nude athymic mice (Ref. 23).

Gurung *et al.* (Ref. 24) analysed the in vitro effects on telomerase of *MST-312*, a chemically modified-derivative of EGCG. ONS76 medulloblastoma cells, and M059K and KNS60 GBM cell lines, were treated with MST-312, resulting in a reduction of TA by about a half. A reduction of proliferation rate was also observed. However, hTERT mRNA and protein levels were not affected; furthermore, MST-312 withdrawal was associated with 95% rescue of basal TA by 72 h. Thus, MST-312 appeared to act as a competitive telomerase inhibitor in brain tumour cells. Given that MST-312 binds to telomere site on DNA, this could activate DNA damage response pathway. Finally, prolonged exposure to telomerase inhibitor was associated with the emergence of resistant sub-populations: this would prompt a strategy of combining telomerase inhibition with DNA repair pathway blockade through DNA-PKcs inhibition.

In another work (Ref. 25), *eribulin* was tested for effectiveness in GBM bearing TERT mutation. TERT may have a telomerase-independent activity as RNA-dependent RNA polymerase (TERT-RdRP), due to its affinity with viral RNA polymerases. The biological functions of TERT-RdRP are unclear; a role in heterochromatin maintenance, in mitochondrial ROS catabolism or in siRNA synthesis has been postulated (Refs 6, 25). Eribulin, originally developed as microtubule inhibitor and currently a FDA-approved treatment of refractory breast cancers, has been found to be a specific inhibitor of TERT-RdRP. In vitro analyses showed significant eribulin-induced growth inhibition in TERT-mutated cells (U87MG, U251MG, U118MG, LN229, GSC23 and GSY03 cells) compared to TERT-wt cells (ES2, TOV21G cells). Orthotopic xenograft of TERT-mutated, luciferase-expressing glioma cells in athymic mice showed reduced growth after eribulin treatment (1.25 mg/kg twice/day) and, accordingly, treated animals had a prolonged survival. Eribulin was found to be able to cross the blood–brain barrier in those areas in which it was altered by the tumour cells, but not the intact blood–brain barrier (Ref. 25).

Compounds acting through hTR inhibition

Marian *et al.* (Ref. 26) focused on the anti-proliferative effect of *imetelstat* (*IMT*, *GRN163L*), a telomerase-specific inhibitor, in GBM-initiating cells. IMT is a short-chain oligonucleotide with high affinity for the template region of the RNA component (hTR or hTERC) of hTERT, which causes a dose-dependent and reversible inhibition of TA. It is currently regarded as the most promising drug inhibiting telomerase, though FDA has not granted marketing license to date. In neurosphere cultures, IMT administration reduced TA after 72 h treatment by 50% at a dose of 0.45 μM, and by 100% at a dose of 4 μM. TA could be recovered in 12 days after drug withdrawal, showing reversible effect of IMT. Prolonged exposure to IMT produced a decrease in clonogenicity by 4 weeks, and subsequently a reduction in proliferation rate and finally cell death (Ref. 26). Moreover, the average telomere length of long-term IMT-treated GBM tumour-initiating cells showed a marked decline from ∼3.5 to <2.0 kilobases. Importantly, pre-treatment with IMT for 72 h was able to enhance the antitumor activity of temozolomide and RT in vitro. In vivo, IMT administration (30 mg/kg i.p. three times per week) was able to reduce TA by 60–70% after 3–5 days of treatment in orthotopic xenografts, and to produce a 10-fold volume reduction in subcutaneous xenografts after prolonged treatment (53 days) (Ref. 26). Ferrandon *et al.* (Ref. 27) evaluated in vivo the therapeutic efficacy of combined IMT and RT in a murine U87MG GBM orthotopic model. After 28 days of treatment, a significant reduction in tumour volume and in TA was showed as compared to controls; moreover, a significant correlation was found between TA and tumour volume reduction. In a subsequent in vivo experiment comparing different treatment regimens (RT alone, IMT alone and RT + IMT), the combination regime was the most effective in prolonging animals survival (Ref. 27). Finally, Ozawa *et al.* investigated in vivo, in orthotopic xenografts of GBM U251 and U87 cells in nude mice, the antitumor effect of hTR inhibitor *GRN163*, an imetelstat analogue (Ref. 28). Intra-tumoural injection of GRN163 resulted in lower tumour growth rate and in significantly improved animals survival.

Compounds acting through shelterin inhibition

Bejarano *et al.* (Ref. 29) validated the antitumor role of telomere-binding protein *TRF1* inhibition. TRF1 is a member of the shelterin complex, which has a key role in telomere protection and in TA. They assayed several *TRF inhibitors* (ETP-47228, ETP-47037, ETP-50946). TRF1 inhibition showed synergistic antitumor effects with γ-irradiation and temozolomide. However, TRF1 inhibitors did not appear to cross blood–brain barrier, thus limiting their clinical applicability.

Compounds acting through G-quadruplex structure stabilisation at 3′ telomere end

Zhou *et al.* studied in vitro the anti-tumour effect of *BRACO-19*, a G-quadruplex (G4) ligand molecule that stabilises telomeric quadruplex DNA structure, on malignant glioma cells (Ref. 30). It has been reported that when the 3′-overhang of telomeric DNA forms a quadruplex structure, it cannot be elongated by telomerase. Therefore, compounds able to stabilise this structure inhibit TA. U87, U251 and SHG44 cells showed dose-dependent cytotoxic effect when treated for 72 h with BRACO-19; on the contrary, human normal astrocytes did not show decreased viability after BRACO-19 treatment, suggesting selective glioma killing. TRAP assay detected decreased TA in glioma cells; moreover, strong phosphorylation of γ-H2AX after 72 h treatment with BRACO-19 was observed, which was also confirmed by the immunofluorescence for γ-H2AX and 53BP1, portending DNA double-strand damage. Confocal microscopy assessed that most of those γ-H2AX and 53BP1 foci were colocalised with TRF1 protein, forming telomere dysfunction-induced foci, demonstrating that BRACO-19 triggered DNA damage at telomeric regions. Cells treated with BRACO-19 displayed typical images of anaphase bridges, which indicated telomere uncapping with dissociation of telomere-binding proteins. These changes led to cell-cycle arrest in G0–G1 phase and apoptotic death. Lagah *et al.* also investigated the ability of G4 ligands to induce telomere uncapping on C6 and U87 glioma cells. *Pentacyclic 3,11-difluoro-6,8,13-trimethyl-8H-quino[4,3,2-kl] a-cridinium methosulfate* (*RHPS4*) appeared to bind to and stabilise G4 DNA isoforms (Ref. 31); this resulted in reduced TA, reduced cell viability and cell-cycle arrest. Thus, both studies demonstrated the anti-proliferative effects of G4 stabilizing molecules on glioma cells through telomerase inhibition.

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Direct gene expression modulation therapies

Gene expression modulation therapies able to reduce TA are listed in Table 2.

Mancini *et al.* (Ref. 32) investigated *GABPβ1L* inhibition as a possible target in GBM cell lines bearing mutant hTERT promoter. The longer isoform of the β1 subunit of GABP (GABPβ1L) induces the constitution of GABP tetramers, which bind to a specific site on the *mutated* hTERT promoter, activating hTERT transcription. Of note, the same tetramer is unable to bind to normal hTERT promoter. The authors used in vitro cultures of lines with mutant TERT promoter (GBM1, T98G, LN229), using lines with wild-type TERT promoter as controls (NHAPC5, HCT116, HEK293T). siRNA- and CRISPR-Cas9-mediated β1L inhibition was associated with a significant reduced TERT expression only in hTERT promoter-mutated lines. In order to further demonstrate dependence on β1L isoform for activation of TERT promoter in TERT-mutant cell lines, the authors obtained post-transcriptional degradation of GABPB1 transcript via locked nucleic acid antisense oligonucleotide; again, β1L knockdown reduced TERT expression across all TERT promoter mutant cultures and had no effect on TERT expression in all TERT promoter wild-type cultures. Mean relative telomere length was significantly reduced in TERT promoter-mutant cells via reduced β1L function. In vivo experiments confirmed the in vitro data, showing reduced tumour growth and prolonged survival in xenograft-bearing mice. Interestingly, while all TERT-mutant cells showed reduced growth and viability in short-term cultures, T98G and LN229 cell clones demonstrated surviving population in long-term cultures, suggesting possible escaping mechanisms to β1L blockade.

hTERT was the most frequent target of gene expression inhibition, mainly using RNA interference techniques. George *et al.* (Ref. 33) investigated the anti-angiogenetic effects *of hTERT inhibition combined with IFN-γ treatment*. This choice was justified by the known interplay between IFN-γ and hTERT, and by the wide range of pathways modulated by IFN-γ. Injection of a recombinant plasmid carrying hTERT siRNA was performed during IFN-γ treatment in SNB19 and LN18 glioma cell lines. In co-treated cells, hTERT mRNA and protein levels appeared reduced and angiogenesis ability was impaired, as shown by in vitro inhibition of capillary-like network formation when co-cultured with endothelial cells. In vivo studies confirmed angiogenesis inhibition and impaired tumorigenic ability by IFN-γ treated cells with hTERT inhibition. WB assay of xenografts showed downregulation of molecules connected to cell invasion (PCNA, MMP), angiogenesis (VEGF, bFGF, CD31) and tumour progression (CDK2, CDK4, cyclin D1). In a series of works conducted by our research group in early 2000s, we investigated telomerase role in modulating GBM aggressiveness by inhibiting hTERT via *hTERT short interfering RNAs* (si1-hTERT, si2-hTERT) in two GBM cell lines, TB10 and U87MG (Refs 8, 34, 35). In vitro, RT-PCR, TRAP and telomere restriction fragment (TRF) assays showed marked reduction of hTERT mRNA, reduction of TA and telomere length reduction in treated GBM cells. However, no difference in cell growth rate was detected between treatment and control groups. Notably, in vivo subcutaneous and orthotopic xenografts demonstrated a markedly impaired tumorigenesis ability by si-hTERT TB10 and U87 GBM cells. Histopathological examination did not detect necrosis in treatment group, while control groups retained massive necrosis; TUNEL assay confirmed increased apoptosis rate in treated xenografts. Moreover, in orthotopic xenografts, a significant reduction of angiogenesis was noticed in the treatment group. The role of telomerase in GBM angiogenesis was confirmed in subcutaneous co-xenografts of human umbilical vascular endothelial cells and TB10 GBM cells: endothelial cells

Table 2. List of direct gene expression modulation therapies for telomerase inhibition

Author (year)	Type of study	Gene modulation strategy	Mechanism of action	Conclusions
Mancini *et al.* (Ref. 32)	*In vitro and in vivo*	siRNA and CRISPR-Cas9-mediated GABPβ1L inhibition	GABPβ1L-containing GABP tetramers are specific transcription factors for mutated hTERT promoter	GABPβ1L blockade causes telomeres shortening and impairs tumour growth
George *et al.* (Ref. 33)	*In vitro and in vivo*	hTERT siRNA + IFN-γ	Interplay between hTERT and IFN-γ	Inhibition of angiogenesis and tumourigenesis. Downregulation of the players involved in cell invasion, angiogenesis and tumour progression
Falchetti *et al.* (Refs 34, 36)	*In vitro* and *in vivo*	hTERT siRNA		Reduction of telomerase activity does not affect cell growth in vitro but inhibits tumour growth in vivo. Survival of endothelial cells require the presence of GBM cells and telomerase reactivation
Lavanya *et al.* (Ref. 36)	*In vitro*	hTERT siRNA		hTERT downregulation decreased cell viability and proliferation rate
Wang *et al.* (Ref. 37)	*In vitro* and *in vivo*	Anti-miR-21 (as-miR-21)	As-miR-21 inhibits STAT3, resulting in hTERT transcriptional blockade and reduction of TA	hTERT and STAT3 inhibition impair tumour growth
Kim *et al.* (Ref. 39)	*In vitro*	shMUC1 (RNA interference for mucin 1 gene)	MUC1 knockdown downregulates hTERT telomerase expression and EMT with unclear mechanisms	*MUC1* knockdown inhibits cell proliferation and resulted in cell cycle arrest at G1 phase. TERT inhibition activated ALT
Vinchure *et al.* (Ref. 40)	*In vitro*	miR-490	TERF2 (shelterin complex player) inhibition	Mir-490 reintroduction blocked telomerase, reduced stemness, induced senescence and activated p53-mediated DNA damage response

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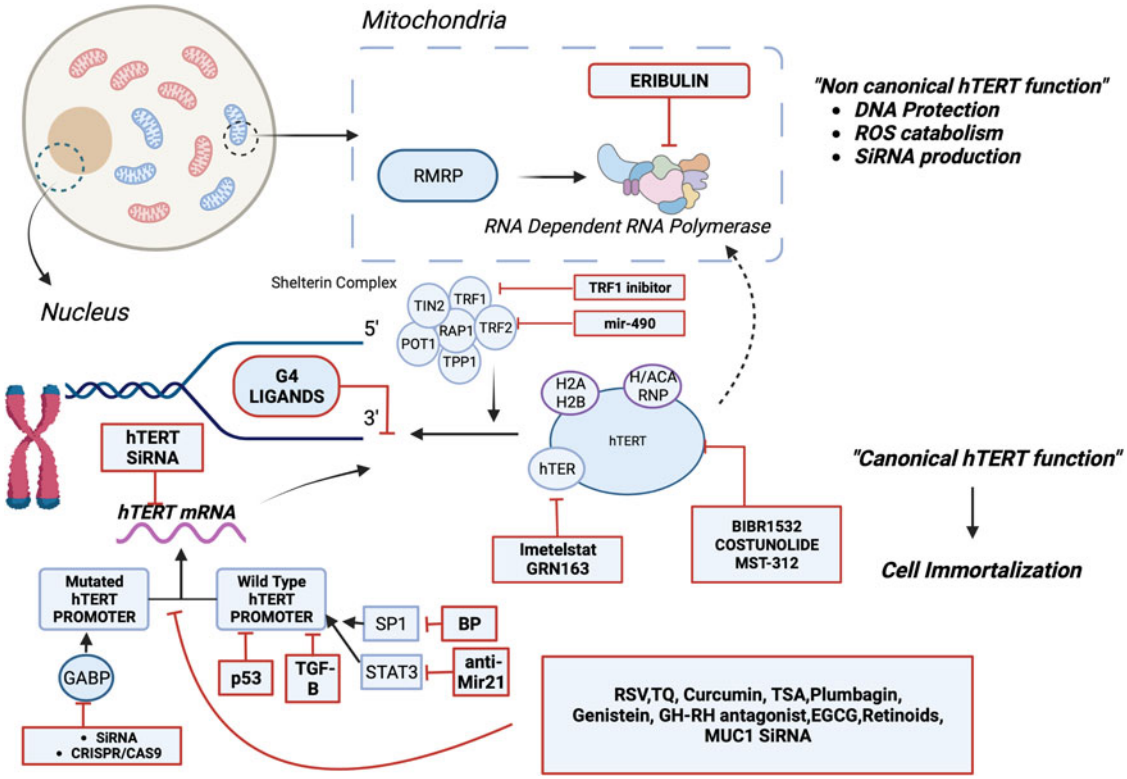

Fig. 2. Telomerase pathway and its inhibitors. BP, butylidenephthalide; EGCG, epigallocatechin gallate; GABP, GA binding protein transcription; H/ACA RNP, H/ACA ribonucleoprotein; hTERT, human telomerase reverse transcriptase; hTER, human telomerase RNA; MUC1, mucin 1; RMRP, RNA component of the mitochondrial RNA-processing endoribonuclease; RSV, resveratrol; TQ, thymoquinone; TSA, trichostatin A. The figure was created in BioRender.com.

survived only when telomerase was upregulated and in presence of tumour cells, while telomerase inhibition strongly reduced their survival (Ref. 35). In a similar experiment, Lavanya *et al.* downregulated hTERT by siRNA in LN18 glioma cell line (Ref. 36). hTERT inhibition was effective in significantly decreasing cell viability at 24 h post-transfection, and annexin V/PI double staining and FACS showed a significant increase in apoptosis levels. The different *in vitro* results obtained in these experiments as compared with those performed by our group are puzzling; probably, as already discussed in previous sections, different GBM cell lines can respond differently to telomerase inhibition.

Anti-microRNA-21 (*as-miR-21*) was used by Wang *et al.* to demonstrate miR-21 positive regulation over hTERT in GBM U87 and LN229 cells (Ref. 37). Firstly, MTT assay and annexin V/PI double staining showed in vitro decreased proliferation and increased apoptotic arrest at G0/G1 cell-cycle phase when GBM cells were transfected with as-miR-21 compared to controls, in association with decreased hTERT mRNA expression by RT-PCR. WB assay highlighted STAT3 decreased expression in as-miR-21 cells, showing a link between STAT3 and hTERT expression. Indeed, STAT3 acts as a positive hTERT transcription factor (Ref. 38). In vivo subcutaneous xenografts confirmed the antitumor role of as-miR-21 and the interplay between hTERT and STAT3.

Kim *et al.* reported the effect of *mucin1* (*MUC1*) suppression in GBM cells (Ref. 39). MUC1 is a single-pass type I transmembrane protein, over-expressed in most human epithelial cancers and involved in epithelial–mesenchymal transition (EMT) and tumour progression. RNA sequencing data of paired normal brain and tumour tissue from 30 patients showed that MUC1 was one of the significantly upregulated genes in glioma, independently of WHO grade. shMUC1 U373 and U87 GBM cells displayed impaired cell proliferation and increased apoptosis. Transcriptome profiling of naive and shMUC1 glioma cells revealed that the main pathways regulated by MUC1 were the EMT and the telomere-related pathway. In MUC1-knockout GBM cells, hTERT expression as well as TA appeared to be reduced, while TRF analyses showed slightly increased telomere length. In fact, MUC1 knockdown induces, as escape mechanism, the ALT pathway, which is characterised by the presence of extrachromosomal telomeric circular DNA. These results suggested that MUC1 depletion contributes to the switch of telomere maintenance mechanism from classic telomerase activation to ALT in GBM cells.

Vinchure *et al.* (Ref. 40) focused on the role of *miR-490*, an oncosuppressor miR epigenetically downregulated in GBM. miR-490 functions as inhibitor of TGF-β-mediated EMT. Moreover, miR-490 modulates the expression of genes involved in telomere maintenance, like TERF2, a member of the shelterin complex. miR-490 reintroduction was effective in shortening telomere length and inducing telomere DNA dysfunction in U87 cell line, harbouring an intact p53, but not in T98G cells, harbouring a mutant p53. miR-490 reintroduction is indeed able to induce DNA damage pathway activation, which in turn triggers p53-mediated anti-proliferative pathways only in cells with intact p53 status.

A graphical summary of all reviewed telomerase inhibitors, both pharmacologic and gene modulation-based, is provided as Figure 2.

Expert and topical summary

Telomerase inhibition raised huge enthusiasm among neuro-oncology scientists, and a variety of inhibition strategies were tested in laboratory, which have been extensively reported in this review. Of note, only a few specific telomerase inhibitors are available: actually, many of the reviewed studies utilise compounds which impair hTERT transcription through unclear

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mechanisms. Since transcriptional hTERT regulation is highly complex (Ref. 38), a number of key pathways are probably modulated by these drugs to obtain hTERT inhibition. This lack of selectivity hinders clinical translation, and, in fact, these efforts have not had the expected clinical impact. Moreover, direct telomerase inhibition strategies carry some conceptual limitations, firstly the long period required before telomeres shortening reach the critically threshold for the activation of senescence and apoptosis machinery (Ref. 30). Furthermore, it has been reported extensively that cancer cells are able to activate a recombination-based ALT mechanism for telomere maintenance, thus jeopardizing telomerase inhibition (Ref. 30). As a consequence, no clinical studies with telomerase inhibitors in GBM can be found in literature. Furthermore, registered trials with telomerase inhibitors in GBM are lacking. A search on clinicaltrials.gov repository revealed only two active, not recruiting, phase 1/2 studies: a French one aiming at testing an anticancer vaccine based on telomerase-derived peptide in newly diagnosed, MGMT-unmethylated GBM patients after concomitant chemoradiation (UCPVax-Glio; clinicaltrials.gov/show/NCT04280848); and a US multicentre study aimed at evaluating a complex treatment, encompassing among others an anti-hTERT DNA plasmid, in newly diagnosed GBM in combination with standard-of-care chemoradiation (clinicaltrials.gov/show/NCT03491683). No results are available for these studies. The situation for other cancers is similar, with no approved anti-telomerase inhibiting treatments. Imetelstat received orphan drug designation for myelofibrosis in 2015, but FDA did not grant approval; the results of ongoing phase III studies could relief this grim scenario (Ref. 41). Our opinion is that novel effective multitarget inhibitors or drug combination strategies are needed, able to block not only telomerase, but also other pathways involved in cell immortalisation and resistance to DNA damage, in order to foster apoptosis machinery activation in a more sustained way. Hopefully, the emphasis given to hTERT mutation in the context of the new WHO classification of central nervous system tumours will rekindle these efforts.

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