Teaser This review reports recent advances in the development of telomerase inhibitors as anticancer agents and highlights the advantages of targeting telomerases in cancer therapy.

Raising the bar in anticancer therapy: recent advances in, and perspectives on, telomerase inhibitors

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Telomerase is a ribonucleic reverse transcriptase enzyme that uses an integral RNA component as a template to add tandem telomeric DNA repeats, TTAGGG, at the 3′ end of the chromosomes. 85–90% of human tumors and their derived cell lines predominantly express high levels of telomerase, therefore contributing to cancer cell development. However, in normal cells, telomerase activity is almost always absent except in germ cells and stem cells. This differential expression has been exploited to develop highly specific and potent cancer therapeutics. In this review, we outline recent advances in the development of telomerase inhibitors as anticancer agents.

Introduction: telomeres and telomerase

Nuclei contain thread-like structures called chromosomes, which carry genetic information from parents to their offspring. Chromosomes comprise tightly coiled DNA bound by proteins called histones, which act as structural support. Interestingly, broken chromosomes are prone to rearrangements and fusion, whereas chromosomal ends are protected from such events. This phenomenon results from the presence of telomeres, often referred to as 'biological clocks'. Telomeres, derived from the Greek words *telos* (ends) and *meros* (parts), are protective ends of linear chromosomes that protect them from unwanted consequences, such as DNA damage, exonucleolytic degradation, recombination, and end-to-end fusion. The length of the telomere is a crucial factor representing the overall health status and viability of the cell. The average length of telomeres differs from species to species. A typical eukaryotic telomere comprises a double-stranded DNA with a guanine (G)-rich sequence, TTAGGG, and associated proteins [1,2]. In addition, it also has a single-stranded 3′ overhang, which folds into the double-stranded telomere and forms the so-called 'T-loop' structure. This T-loop structure has a protective role and is

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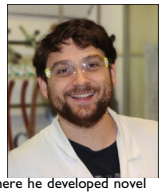

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responsible for capping the chromosomes, thus preventing the loss of genetic information. Also, because of the high G content, the single-strand overhang can lead to the formation of G-quadruplexes (G-4), where G can assist in hydrogen bond formation, being both a hydrogen bond acceptor and donor (Fig. 1a). The T-loop structure is stabilized by a group of six proteins called the shelterin complex, encompassing telomere repeat factor 1 and 2 (TRF1 and TRF2), protection of telomere 1 (POT1), TRF1-and TRF2-interacting nuclear protein 2 (TIN2), TINT1/PTOP/PIP1 protein (TPP1), and repressor/activator protein 1 (Rap1) [3]. TRF1, TRF2, and POT1 bind directly to the telomeric repeats, whereas TRF1 and TRF2 bind to the double-stranded DNA [4,5]. However, POT1 binds to the single-stranded overhangs [6].

During each round of cell division, there is an incomplete replication of telomeres, leading to telomere shortening, referred to as the 'end-replication problem' [7,8]. This results from the inability of DNA polymerases to perform complete replication of linear DNA templates. Telomere shortening occurs because of the loss of 50–200 nucleotides per round of replication. At a certain critical length (4–6 kb), the continued shortening is detected as DNA damage, resulting in cell death events, such as senescence or apoptosis (Fig. 1b), termed the DNA damage response (DDRs), resulting from the formation of telomere dysfunction induction focus (TIF) [9]. Proliferative cells use telomerase as a replenishing tool to overcome the end replication problem [10].

Telomerase is a ribonucleoprotein present in mammalian cells that adds TTAGGG tandem repeats to the 3′ end of the telomere [11]. It was first examined in the transformed cervical carcinoma (HeLa) cell line in 1989 [12]. It comprises three components: (i) the catalytic subunit human telomerase reverse transcriptase (hTERT), responsible for the addition of nucleotides to the 3′ end; (ii) a 451-nucleotide-long human telomerase RNA (hTER), which acts as an RNA primer; and (iii) associated proteins, including human telomerase-associated protein 1 (hTEP), protein 23 (p23), heat shock protein 90 (Hsp90), and dyskerin. Being a reverse transcriptase, the hTERT subunit utilizes the template portion (3′-CAAUCCCAAUC-5′) of hTER to synthesize the telomeric DNA. Besides telomere maintenance, telomerase also has a role in other functions, such as gene expression regulation, cell proliferation, apoptosis, Wnt/β-catenin signaling, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling, MYC-driven oncogenesis, cell adhesion, cell migration, and epithelial-mesenchymal transition (EMT) [13]. The 2009 Nobel Prize in Physiology or Medicine was awarded to Elizabeth H. Blackburn, Carol W. Greider and Jack W. Szostak for their influential work on telomere biology and biochemistry.

G-quadruplexes

Accumulating data indicate that, in addition to antiparallel double helices, DNA can form a variety of less common secondary structures, of which G-4 are among the most significant [14,15]. These structures have been observed in many DNA sequences, including important oncogenes (*c-myc*, *c-kit*, etc.) [16], centromeres, and the single-stranded G-rich overhang of telomeres [17,18]. In general, the G-rich nucleic acid sequences tend to adopt the G-4 conformation, represented by G-quartets (also named G-tetrad) stacking on top of each other. G-quartets are planar structures of four Gs linked together by hydrogen bonds. G-4 can adopt many conformations depending on the nature of the loop, such as strand stoichiometry, strand polarity, glycosidic torsion angle, and location of the loops that link the quinidine strand(s). They can be folded from a single G-rich sequence intramolecularly or intermolecularly from the association of two or four separate strands, leading to the formation of dimeric or tetrameric G-4 structures, respectively. The presence of eight carbonyl groups in the hole between G-tetrads generates a strong negative electrostatic potential in the central cavity of G-4, which enables these structures to coordinate cations [19]. Two intramolecular G-4 structures have been identified for telomeric sequences, depending upon the incubation conditions. In Na^+ solution, nuclear magnetic resonance (NMR) analysis revealed an antiparallel basket-type conformation with diagonal loops. In the presence of K^+, a propeller-type conformation (showing no loop regions and a parallel arrangement) has been reported [19–21]. The stabilization of telomeres into G-4 structures by small ligands can lead to telomerase inhibition or shortening of telomere length, induction of senescence, inhibition of cell growth, and end-to-end fusion of chromosomes. Some G-4 ligands can prompt dissociation of the telomere-binding proteins POT1 and TRF2, thus uncapping telomeres to make them available for extension [19,22–24].

Many other important G-rich containing sequences are included in *c-myc*, *c-kit* and *bcl-2* genes. *c-myc* and *c-kit* are two oncogenes linked to a range of human cancers and involved in many cellular events, such as apoptosis, differentiation, and adhesion. *Myc* genes are frequently deregulated in cancer and the overexpression of *c-myc* leads to telomerase reactivation and telomere stabilization, which can eventually result in limitless proliferation [24]. The stabilization of G-4 within *c-myc* and *c-kit* can suppress their transcriptional activation and downregulate their expression. The *bcl-2* proto-oncogene leads to the expression of the Bcl-2 related protein. This protein is involved in the control of programmed cell death, functioning as an apoptosis inhibitor. In many human cancers that overexpress Bcl-2, targeting the G-4 of *bcl-2* with stabilizing ligands can lead to apoptosis [19].

Telomerase as a target for cancer therapy

Cancer is a group of diseases that involve the abnormal growth of cells in an uncontrolled manner that can spread, metastasize, and invade other tissues. Cancer remains a serious life-threatening disease with high morbidity and mortality. The risk of developing cancer increases with age and important factors for cancer development include genetic issues, lifestyle, environmental factors, and smoking. According to the WHO, an estimated 18 million patients worldwide would be diagnosed with cancer in 2018, and around 9.6 million patients worldwide were estimated to die in 2018 based on Global Health Observatory (GHO) data [25].

The telomerase enzyme is upregulated in 80–90% of cancer cells and has been observed in various malignancies, such as breast, colon, melanoma, ovarian, pancreatic, oral, prostate cancers, and in cancers of soft tissues [26,27]. hTERT activation or upregulation is a significant feature in the proliferation of cancer cells. Recent studies identified cancer-specific mutations in two sites within the promoter region of hTERT as a possible mechanism for activation of telomerase in cancers. The two C → T mutations occur at −124 base pairs (bp) and −146 bp upstream from the ATG site [28,29].

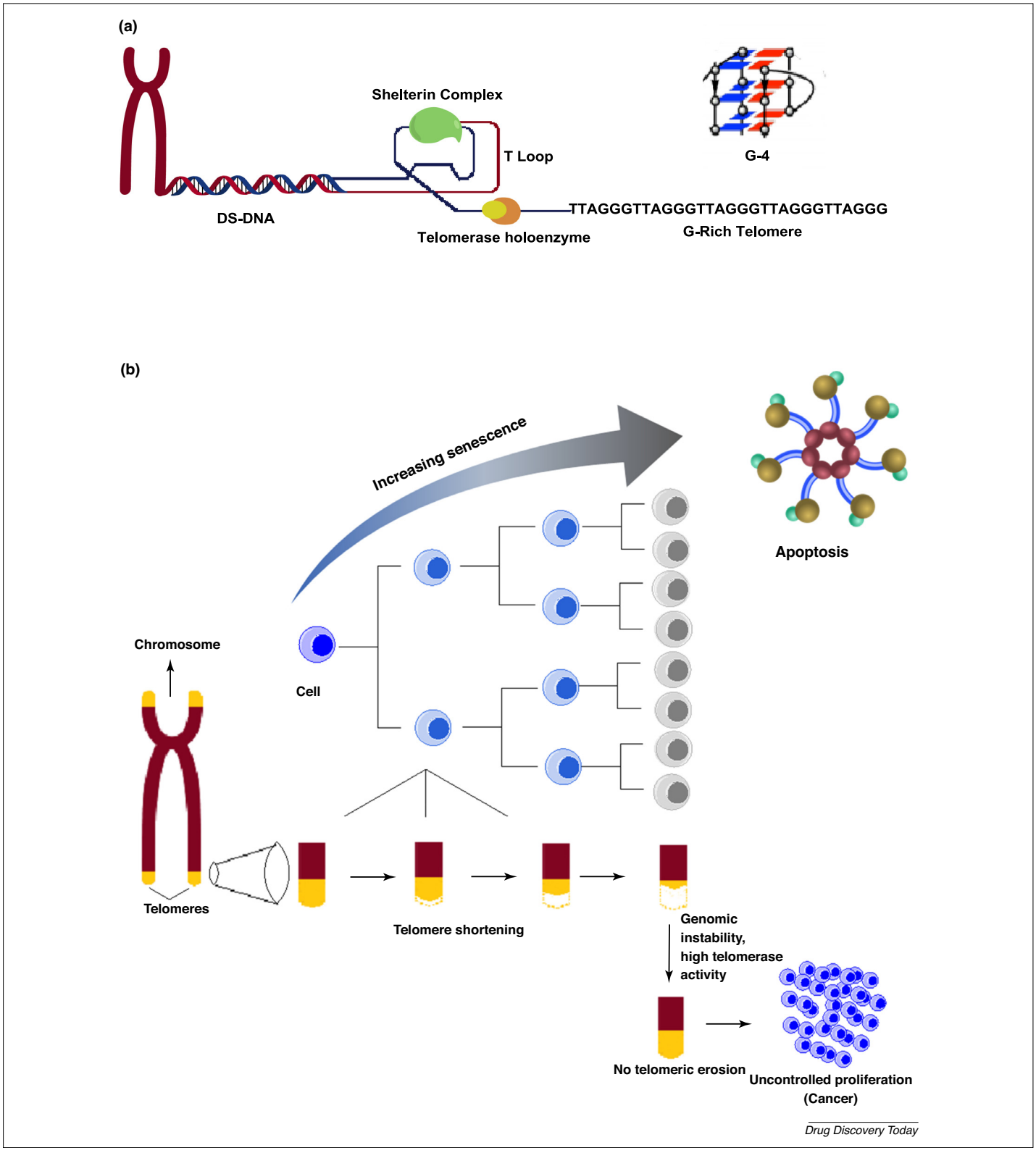

FIGURE 1

Telomere length as a critical factor in cellular fate. (a) Representative figure of a typical eukaryotic telomere with a double-stranded DNA (DS-DNA) in association with the shelterin protein complex. Telomere structure also contains a guanine (G)-rich overhang folded into the double-stranded DNA to form the protective T-loop structure. A high G content leads to the formation of G-quadruplexes (G-4) that serve as both hydrogen bond donors and acceptors. (b) Representative figure depicting telomeric length as a crucial factor determining cellular fate. Cellular fate depends upon telomere length and can follow either of the two alternative paths: normal (i) and cancer (ii). Shortening of telomere length to a crucial length (4–6 kb) is detected as DNA damage, ensuing DNA damage responses, such as senescence and apoptosis ('normal path'). By contrast, high telomerase activity can lead to maintenance of telomere length, characterized by the uncontrolled proliferation of cells, as observed in various malignancies ('cancer path').

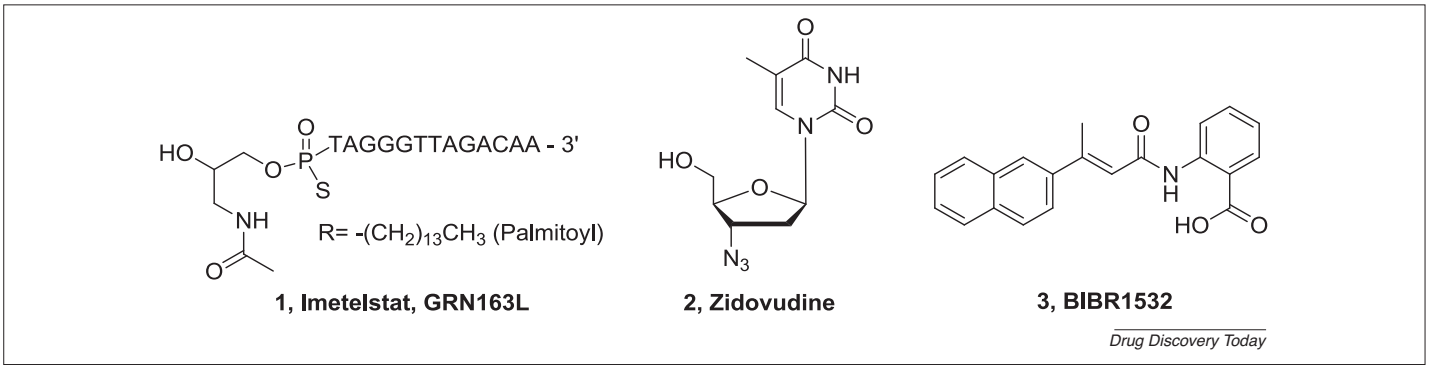

FIGURE 2

Representative structures of well-known telomerase inhibitors (compounds **1**–**3**).

Telomerase expression can also depend on other factors, which could be transcriptional, post-transcriptional, or epigenetic in nature [30–32]. However, the exact mechanisms responsible for high levels of telomerase activation in cancer cells are under investigation and are still not completely clear. Alternative lengthening of telomeres (ALT) is another form of homologous DNA recombination-mediated replication, observed in 10–15% of cancers. This is a mechanism used by proliferative cells to bypass senescence [33] and is predominant in osteosarcomas and astrocytic brain tumors [34].

Telomerase has been validated as an effective drug target and biomarker in cancer because of its increased expression in cancer cells [35,36]. Telomerase activity is higher in advanced and metastatic tumors, whereas in normal cells, it is minimal or undetectable except in germ line cells, stem cells, and other cells, such as cardiovascular cells, where it is detectable, but quiescent in nature. The telomeric repeat amplification protocol (TRAP) was developed in 1994 to measure telomerase activity in mammalian cells and tissue samples. It is performed in three steps: extension, amplification, and detection of telomerase. The extension step involves the addition of telomeric repeats to a telomeric substrate (TS) oligonucleotide (mimicking the 3′ telomere end) using a cell lysate containing telomerase. In the amplification step, polymerase chain reaction (PCR) is used to amplify the extension products using specific primers followed by detection using electrophoresis. The TRAP assay is a highly sensitive protocol that can detect as few as 0.01% telomerase-positive cells in a mixed population. Given its high sensitivity, robustness, and reproducibility to detect and quantify telomerase activity in both tumor and normal cells, it helps endorsing telomerase as an effective anticancer target [37]. Inhibition of telomerase activity prompts telomeric DNA erosion, which then leads to the formation of dysfunctional telomeres initiating TIF formation. Given the lack of telomerase activity, once cancer cells reach the critical length of telomere shortening, they fail to form the protective T-loop structure because of improper folding. Loss of functional telomeres in cancer cells induces apoptosis, whereas the loss of telomeres in normal cells leads to cell cycle arrest.

Current therapies involving telomerase inhibitors

Exploiting the differential activity of telomerase in normal and cancer cells is vital for the development of novel anticancer agents [38]. Several approaches have been identified to inhibit telomerase activity, such as: (i) targeting telomerase reverse transcriptase catalytic activity; (ii) targeting telomerase–RNA interactions; (iii) directly targeting the telomerase RNA component; and (iv) targeting dyskerin in ribonucleoprotein biogenesis [36]. Over the past three decades, several telomerase inhibitors have been developed and evaluated for their anticancer potential (Fig. 2).

These inhibitors include chemically modified oligonucleotides (e.g., imetelstat, **1**), nucleoside analogs (e.g., zidovudine, **2,** AZT), small molecules as synthetic mixed-type noncompetitive non-nucleoside analogs (e.g., BIBR1532, **3**), natural compounds (rhodocyanin, curcumin, and genistein), G-4 stabilizers (telomestatin) and vaccines. Imetelstat (**1**), one of the most successful telomerase inhibitors synthesized to date [39], is a 13-mer *N*3′,*N*5′-thiophosphoramidate (NPS) oligonucleotide containing a covalently bound 5′-palmitoyl (C16) lipid group. The nucleic acid backbone provides resistance against cellular nucleases, thus improving stability in tissues and plasma as well as the binding affinity to the target. The lipid group increases cell permeability, which enhances the potency and contributes to the improvement of pharmacokinetic (PK) and pharmacodynamic (PD) profiles. It has a long residence time in spleen, liver, and bone marrow. Moreover, it binds to the template of an RNA component of telomerase with a high affinity, resulting in direct and competitive telomerase inhibition. The preclinical studies on imetelstat showed that it can inhibit the proliferation of both solid and haematological tumors in cell cultures and rodent xenograft models. It successfully inhibits malignant progenitor cells from hematological cancers, such as acute myeloid leukaemia (AML) and multiple myeloma [40,41]. It exhibits an additive or synergistic effect in combination with approved anticancer drugs in a variety of cell culture systems and xenograft models. Phase I studies in patients with advanced solid tumors, multiple myeloma, and chronic lymphoproliferative diseases with imetelstat as a single agent and in combination with other anticancer agents (e.g., bortezomib, paclitaxel, bevacizumab, and carboplatin) showed telomerase inhibition in various tissues. Currently, imetelstat is under investigation in two clinical trials involving patients with myelofibrosis (Phase II) and myelodysplastic syndromes (Phase II/III). Several other telomerase inhibitors, summarized in Table 1, are under clinical investigation. Zidovudine (**2**, AZT), a nucleoside analog originally used for the treatment of retroviral infections, can inhibit telomerase both *in vitro* and *in vivo* and leads to telomere shortening and cell cycle perturbations in cultured cells

TABLE 1

Telomerase inhibitors under clinical investigation[a,b]

Agent	NCT identifier	Phase	Condition	Status
Imetelstat	NCT01568632	1	Solid tumors; lymphoma	Withdrawn
	NCT01916187	1	Neuroblastoma	Withdrawn
	NCT01256762	2	Locally recurrent or metastatic breast cancer	Completed
	NCT01242930	2	Multiple myeloma	Completed
	NCT01137968	2	NSCLC	Completed
	NCT01243073	2	Essential thrombocythemia; polycythemia vera	Completed
Imetelstat sodium	NCT01273090	1	Brain and central nervous system tumors; lymphoma; lymphoproliferative disorder; small intestine cancer; unspecified childhood solid tumor	Completed
	NCT01836549	2	Anaplastic astrocytoma; anaplastic ependymoma; astrocytoma, grade II; ependymoma; giant cell glioblastoma; glioblastoma; gliosarcoma; oligodendroglioma; brainstem tumors	Terminated
PAMIR01	NCT02842333	NA	Chronic leukemia	Recruiting
UCP	NCT02838433	NA	Melanoma	Unknown
	NCT02846103	NA	Lung cancer	Recruiting

[a] All the data from https://clinicaltrials.gov/.
[b] Abbreviations: NA, not applicable; UCP, universal cancer peptides, derived from human telomerase (hTERT).

[42]. BIBR1532 (**3**) is a synthetic mixed-type noncompetitive nonnucleoside telomerase inhibitor (IC_{50} = 93 nM) that specifically targets the telomerase catalytic subunit, hTERT. Its exact mechanism of action is still unclear, but it could block telomerase translocation. BIBR1532 causes apoptosis and senescence in cancer cells [43]. However, it shows limited bioavailability and induces cytotoxicity only when administered in high doses.

Despite significant advances in highly sensitive assays and instrumentation to identify and screen telomerase inhibitors, only a few inhibitors have been made available to the clinic. In this review, we outline recent advances in drug discovery pertaining to telomerase inhibitors and highlight the potential of using telomerase-based therapies to develop specific and potent anticancer agents.

Small molecules as telomerase inhibitors

Given the immense potential of inhibiting telomerase, much research effort has been focused on the design and synthesis of potential telomerase inhibitors as anticancer agents. This has involved exploiting various approaches, such as high-throughput screening (HTS) [44], structure-based drug design, and natural product [45] inspired drug discovery to find new lead molecules, which could pave the way to the development of telomerase inhibitors with optimized drug-like profiles. The small-molecule inhibitors reviewed in this article bind to the active site of hTERT catalytic subunit, which has been examined using two crystal structures: Protein Data Bank (PDB) 3DU6 and the hTERT-BIBR1532 complex [43]. Structurally, the compounds can be clustered based on their core scaffold, where the most common moieties are coumarins, dihydropyrazoles, chromenes, 5-membered heterocycles (oxadiazole, imidazole, and pyrazole), and others (Fig. 3).

Novel coumarin-dihydropyrazole derivatives were developed as antiproliferative agents and were screened against four cell lines: MGC-803 (gastric carcinoma), BCAP-37 (human papillomavirus-related endocervical adenocarcinoma), SGC-7901 (gastric carcinoma); and HEPG-2 (hepatoblastoma) cell lines *in vitro*. Among them, compound **4** (Fig. 3a) was found to be the most potent and demonstrated high inhibitory activity against the HEPG-2 cell line. To study the selectivity against tumor cells versus human somatic cells, a proliferative inhibition assay was performed involving normal gastric mucosa cells (GES-1) and human normal liver cells (L-02). Compound **4** was found to be nontoxic to both normal cells lines, indicating a high selectivity index towards tumor cells. Its telomerase inhibition activity was measured using HEPG-2 cell extracts, in which strong inhibition was registered (IC_{50} = 0.98 ± 0.11 μM). Cell cycle analysis showed that derivative **4** caused cell cycle arrest in G0/G1-phase. Interestingly, it was found to exert its antiproliferative activity by modulating hTERT through the Wnt/β-catenin signal pathway [46]. In addition, a promising class of novel coumarin-dihydropyrazole thioethanone derivatives was designed and synthesized as potential telomerase inhibitors. Their antiproliferative activity was evaluated against several cell lines, including MGC-803, BCAP-37, SGC-7901, and HEPG-2. Compound **5** was found to be the most potent among a series of analogs with an IC_{50} of 0.92 ± 0.09 μM. Moreover, compound **5** induced cell cycle arrest in the G0/G1-phase [47]. Another class of novel dihydropyrazole derivatives was synthesized as telomerase inhibitors using a structure-based drug design approach. Several compounds showed potent anticancer activity against the MGC-803 cell line *in vitro* with low toxicity in normal cells. Among them, derivative **6** was the most potent telomerase inhibitor, with an IC_{50} of 0.98 μM, determined using a modified TRAP assay. *In vivo* studies indicated that compound **6** could inhibit the growth of HEPG-2 and S180 tumor-bearing mice [48]. Coumarin-pyrazoline derivatives with telomerase inhibitory potential were developed and initially screened for their anticancer activity against HEPG-2 cells using an MTT viability assay, which showed that most of the compounds had antiproliferative activity with an IC_{50} range of 10–99 nM. The best-performing telomerase inhibitors were selected by means of the TRAP-PCR-ELISA assay, and compound **7** was found to be the most potent inhibitor, also endowed with significant proapoptotic effects [49]. 2-Phenylpyrimidine coumarin derivatives were developed as potential telomerase inhibitors and their *in vitro* antiproliferative activity was determined against CNE2 (nasopharyngeal carcinoma), KB (human papillomavirus-related endocervical adenocarcinoma), and Cal27

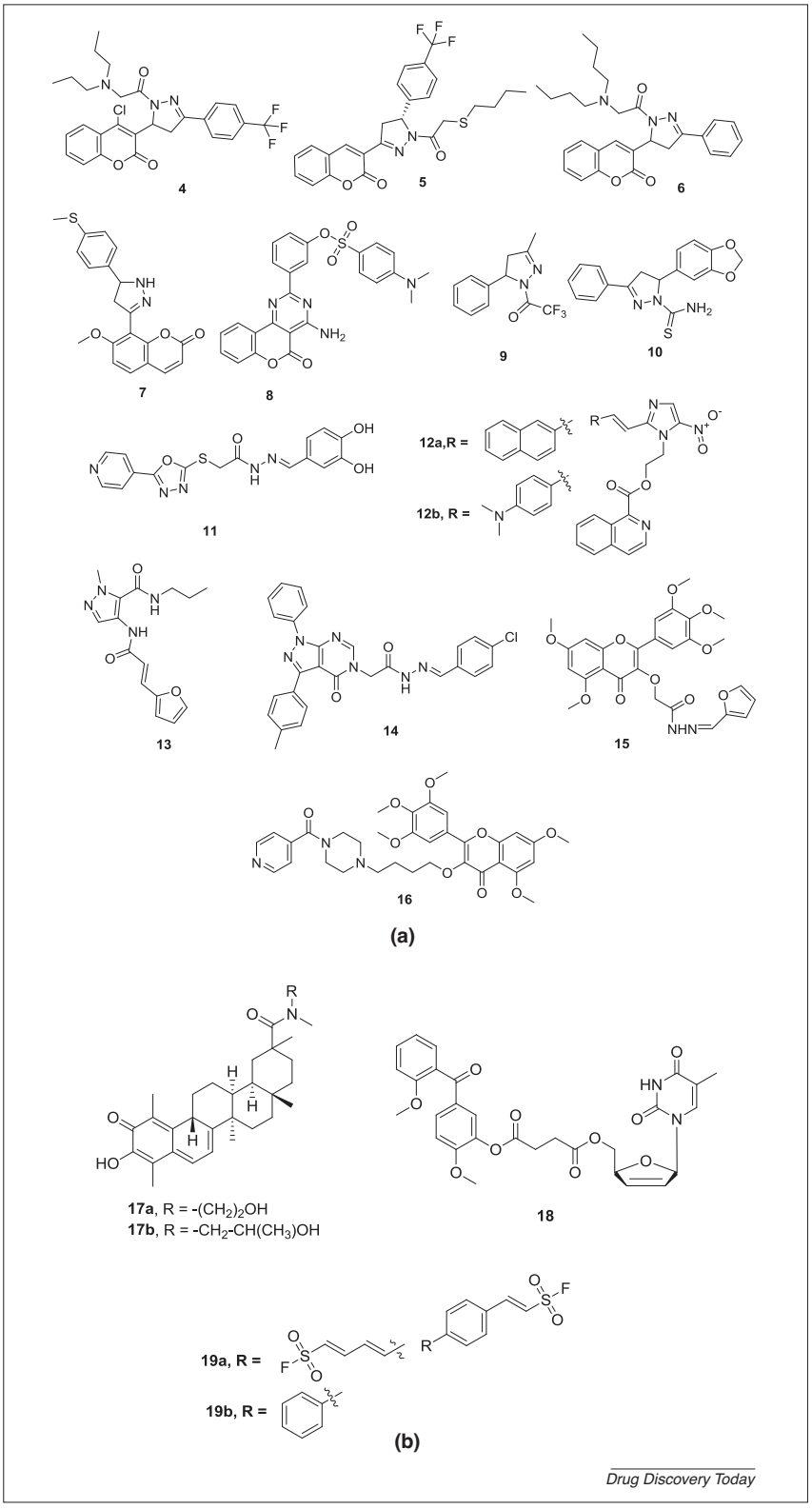

FIGURE 3

Representative structures of small-molecule telomerase inhibitors: (a) compounds **4**–**16** and (b) compounds **17**–**19**.

(tongue squamous cell carcinoma) cell lines. Most of the compounds were active, with derivative **8** being the most potent against CNE2 cells, exhibiting a strong telomerase inhibitory activity with IC_{50} of 0.82 ± 0.14 μM. Flow cytometry analysis indicated that compound **8** induced apoptosis in CNE2 cells. In addition, compound **8** also reduced telomere length, determined using telomerase restriction fragment (TRF) experiments [50].

N-Acyl-4,5-dihydropyrazole derivatives were also developed as potential telomerase inhibitors. Their antiproliferative activity was determined against three cell lines: SGC-7901, HEPG-2, and MGC-

803. Among them, compound **9** exhibited an EC_{50} of 2.06 ± 0.17 and 2.89 ± 0.62 μM against SGC-7901 and MGC-803, respectively, which was better than that of the currently used chemotherapeutic agent 5-fluorouracil. Compound **9** inhibited telomerase with an IC_{50} value of 1.86 ± 0.51 μM determined by using a modified TRAP assay with MGC-803 cell extracts [51]. A novel class of 4,5-dihydropyrazole derivatives containing an oxygen-bearing heterocycle was also developed. Compound **10** was the most potent and demonstrated good activity against the human gastric cell line SGC-7901, with an IC_{50} value of 10.95 ± 0.60 μM, and 0.6 μM inhibition potency for telomerase activity determined using a modified TRAP assay. Moreover, flow cytometric analysis and western blotting showed that inhibitor **10** was able to induce both apoptosis and autophagy [52].

Further published reports indicate that novel 1,3,4-oxadiazole derivatives containing pyridine and acyl hydrazone moieties have also been developed as telomerase inhibitors. Compound **11** was the most potent, with broad-spectrum anticancer activity against HEPG-2, MCF7 (breast adenocarcinoma), BGC823 (gastric adenocarcinoma), and SW1116 (colon adenocarcinoma) cell lines, with an IC_{50} ranging from 0.76 μM to 1.54 μM. Telomerase inhibitory activity of compound **11** was found to be the highest with an IC_{50} of 1.18 ± 0.14 μM [53]. An additional report described a series of 1-quinoline-2-styryl-5-nitroimidazole derivatives synthesized as telomerase inhibitors. Their antiproliferative potential was screened against four cell lines: U251 (astrocytoma), HeLa (human papillomavirus-related endocervical adenocarcinoma), HEPG-2, and A549 (lung adenocarcinoma). Most of the compounds demonstrated potent *in vitro* inhibitory activity in both enzymatic and cellular assays. The analogs **12a** and **12b** were found to be the most potent of the series, with IC_{50} of 0.98 and 1.92 μM, respectively [54]. Pyrazole-5-carboxamide and pyrazolo-pyrimidine derivatives were additionally developed and their antiproliferative potential was tested against the MGC-803, SGC-7901, and BCAP-37 cell lines. Among them, compound **13** displayed potent inhibitory activity of MGC-803 cell growth and showed telomerase inhibition activity with an IC_{50} of 1.02 ± 0.08 μM. Flow cytometry experiments showed that inhibitor **13** arrested cell cycle in the S-phase [55]. A series of pyrazolo[3,4-*d*]pyrimidines were also identified as potential telomerase inhibitors. Compound **14** demonstrated high inhibitory activity against Ehrlich ascites carcinoma cells (EAC) with IC_{50} of 39 μM. It also showed telomerase inhibitory activity with IC_{50} of 30 μM determined using the TRAP assay [56].

Myricetin, a polyphenolic flavonoid compound, is present in a variety of fruits and exhibits anticancer properties via the induction of apoptosis [57]. Based on this, novel derivatives of myricetin were developed as telomerase inhibitors. Among the synthesized compounds, derivative **15** displayed the most potent inhibitory activity, with an IC_{50} of 0.9 μM. Compound **15** exhibited the highest anticancer activity against the human breast adenocarcinoma cell line MDA-MB-231. Flow cytometric results indicated that it caused cell cycle arrest at the G0/G1-phase. Moreover, it was found to also regulate the expression of p65 and hTERT proteins [58]. Novel trimethoxyphenyl-4*H*-chromene derivatives were synthesized as potential inhibitors of telomerase through regulation of dyskerin. *In vitro* antiproliferative studies demonstrated that compound **16** displayed the best potency against HeLa, SMMC-7721 (human papillomavirus-related endocervical adenocarcinoma), SGC-7901, U87 (glioblastoma), and HEPG-2 cell lines with IC_{50} of 1.02, 1.33, 1.35, 2.50, and 4.12 μM, respectively. Derivative **16** inhibited telomerase activity in SGC-7901 cell extract with an IC_{50} of 0.89 μM and also inhibited the expression of dyskerin, as determined by western blotting and immunofluorescence detection. It also induced apoptosis by regulating dyskerin and caused cell cycle arrest in SGC-7901 cells. *In vivo* studies involving diethylnitrosamine-induced hepatic tumors in rats showed improved pathological changes in rats with hepatic tumors. The hepatic cells showed reduced atypia, high differentiation, and full cytoplasm [59].

Celastrol is a pentacyclic triterpenoid isolated from the roots of *Tripterygium wilfordii* and belongs to the family of quinone methides. It is a bioactive compound known to exhibit beneficial effects against a variety of cancers, including pancreatic, prostatic, and squamous cancers, both in *in vitro* and *in vivo* [60,61]. Celastrol derivatives were developed as potential telomerase inhibitors and showed potent anticancer activity *in vitro* against the SGC-7901, SMMC-7721, HEPG-2, and MGC-803 cell lines. Among these, derivatives **17a** and **17b** (Fig. 3b) were the most potent, with high antiproliferative activity with IC_{50} of 0.10–1.22 μM. The presence of hydrophilic moieties in **17a** and **17b** was thought to be the reason for their high activity. Compound **17a** significantly induced apoptosis in SMMC-7721 cells. Telomerase inhibition was determined using a modified TRAP assay and both **17a** and **17b** exhibited high inhibitory activity, with IC_{50} of 0.11 and 0.34 μM, respectively [62].

Phenstatin-linked stavudine derivatives were developed as a novel class of potential telomerase inhibitors. Their antiproliferative activity was determined against four cell lines: HeLa, SMMC-7721, SGC-7901, and HEPG-2. Compound **18** was found to be the most potent against HeLa, SMMC-7721, and SGC-7901 cells, with IC_{50} of 1.58 ± 0.20, 0.82 ± 0.11, and 0.77 ± 0.33 μM, respectively. Compound **18** demonstrated excellent telomerase inhibitory activity with IC_{50} of 0.77 μM against SGC-7901. In addition, it induced cell cycle arrest at the G2-phase and induced apoptosis of SGC-7901 cells. *In vivo* studies involving murine sarcoma cells (S180) and hepatoma cells (HEPG-2) were performed, and treatment with **18** resulted in a decrease of tumor weights in S180 and HEPG-2 tumor-bearing mice [63]. Ethenesulfonyl fluoride derivatives were developed as potential telomerase inhibitors by using a structure-based design. Among them, compound **19a** was the most potent derivative against A375 (amelanotic melanoma) and MDA-MB-231 cell lines with IC_{50} of 1.58 and 3.22 μM, respectively, and was devoid of toxic effects on GES-1 and L-02 normal cell lines, with IC_{50} values <2 mM. Telomerase inhibitory activity was measured using a modified TRAP assay using A375 cells, and compound **19b** exhibited potent telomerase inhibitory activity, with an IC_{50} of 0.71 μM [64].

G-quadruplex stabilizers as telomerase inhibitors

Given that G-4 structures have a large π-surface, small molecules that can interact with G-4 have a large π-surface to maximize the π–π interaction between these structures. Given the negative charge in the central zone of the G-4, metal complexes can be perfect stabilizers of these sequences. Moreover, a metal ion coordinated to heteroaromatic ligands with a square-planar geometry

can lead to better G-4 stabilization. Indeed, the more electropositive metal ion will replace the K^+ or Na^+ cations that normally occupy the center of the G-4, leading to stronger electrostatic stabilization (resulting also from the electron-withdrawing properties of the metal), which reduces the electron density on the coordinated ligand, leading to a system that can display stronger π–π interactions [14]. To quantify the stabilization and selectivity of quadruplex ligands, the fluorescence resonance energy transfer (FRET) assay can be used for different G-4 topologies. Generally, the stabilization values depend on parameters such as fluorescent tags, and incubation buffer; the method for calculating the melting temperature (ΔT_m) denotes the increase in the melting temperature of the G-4 induced because of the presence of ligand [65]. Given our increased understanding of the relationship between G-4 stabilization and its effect on many biological processes, several promising G-4 stabilizers have been synthesized. Here, we outline the efforts of various groups to develop G-4 stabilizers and their telomerase inhibition potential.

Platinum-based inhibitors

In a recent report, two propeller-shaped, trigeminal-ligand-containing, flexible trinuclear Pt(II) complexes were developed. The complex {[Pt(dpa)]$_3$(ptp)} $(NO_3)_6$ (dpa and ptp are *bis*-(2-pyridylmethyl)amine and 6′-(pyridine-3-yl)-3,2′:4′,3″-terpyridine respectively) was found to be a potent G-4 stabilizer. A FRET assay established its ability to increase ΔT*m* of some G-4s (the most sensitive is the hTel G-4) up to 15 °C. In the TRAP assay, compound **20** (Fig. 4a) demonstrated an IC_{50} of 4.2 μM against human telomerase [66]. In another study, the activity of *cis*-[$PtCl_2$(lirioidenine)(DMSO)] **21** was analyzed and compared with the free ligand and cisplatin. Complex **21** (Fig. 4a) showed potent *in vitro* anticancer activity with an IC_{50} of 6.9 μM against the BEL-7404 (human papillomavirus-related endocervical adenocarcinoma) cell line and caused both G_2/M and S-phase cell cycle arrest as well as apoptosis induction via the mitochondrial pathway. It was also able to stabilize G-4-F21 T in the FRET-melting assay by increasing its melting temperature by 14.7 °C and displayed an inhibition ratio in hTERT of 62.5% at a 2 μM concentration [67]. Two oxoisoapopine-organoplatinum (II) complexes were also prepared and evaluated for their anticancer activities. Compound **22** demonstrated excellent G-4 stabilization and telomerase inhibition. A FRET-melting assay established an increase of +14.5 °C in the melting point of the FPu18 T G-4 after treatment at a 1.5 μM concentration. TRAP displayed telomerase inhibition with an IC_{50} of 0.90 μM. It was also observed that complex **22** could arrest cell growth at the G1 = phase and induced apoptosis. Its *in vitro* activity was investigated in various cell lines, including T-24 (bladder carcinoma), HEPG-2, SK-OV-3 (ovarian serous cystadenocarcinoma), SKOV-3/DDP (epithelial ovarian cancer), BEL-7404, NCI-H460 (large cell lung carcinoma), and HCT-8 (colon adenocarcinoma), and showed an IC_{50} range from 4.5 μM to 30.5 μM. Moreover, **22** demonstrated high safety *in vivo* and a high inhibitory effect on tumor growth in HCT-8 and NCI-H460 xenograft mouse models (43% and 50%, respectively at 6 mg/kg dose), thus performing better than cisplatin [68]. The same research group described the antitumor activity of a novel Pt(II) complex using 2-(4-methoxyphenyl)imidazo[4,5-*f*][1,10]phenanthroline and 1,3-propanediamine as the ligand (**23**). The IC_{50} was evaluated against several cancer cell lines, showing the highest potency in HeLa, SK-OV-3, and BEL-7402 cell lines in a range of 9.7 μM to 35.8 μM. The thermodynamic stability of the G-4 in the presence of a 2 μM concentration of **23** was also evaluated, leading to a ΔT_m of +12 °C in FPu18T G-4. The TRAP assay showed a clear decrease in the activity of the telomerase, probably because of the interaction with *c-myc* G-4. It was also observed that complex **23**, at a 10 μM concentration, induced cell cycle arrest at S-phase and apoptosis in HeLa cells by triggering caspase-3/9 activity [69].

Two porphyrin-bridged tetranuclear Pt(II) complexes were synthesized and their activity against telomerase was evaluated. A FRET-melting assay showed that compound **24** (Fig. 4b) raised the melting temperature of G-quadruplexes (ΔT_m +22.4 to +37.8 °C at 0.8 μM) especially for human telomere G-4 (HTG21). To confirm the telomerase inhibition, a TRAP assay was performed, which showed an IC_{50} value of 0.25 μM. The cytotoxicity of **24** was measured against HeLa, HEPG-2, CNE-2, and A549 cancer cell lines, providing IC_{50} values ranging from 23.5 μM to 50.5 μM. This complex was also shown to have the ability to kill cancer cells by inducing apoptosis and causing G2/M-phase arrest when tested at a 40 μM concentration in HeLa cells [70]. In a further report, a Pt complex with 9-amino-oxoisoporphine (**25**) was synthesized and its pharmacological properties were explored. It was found that **25** acts as a telomerase inhibitor with an inhibitory rate of 57% at a 10 μM concentration on HEPG-2 cells, because of the interaction with the *c-myc* G-4-DNA. It also triggered apoptosis in HEPG-2 cancer cells and led to both G2/M-phase and S-phase cell cycle arrest. The cytotoxicity of this complex against different cancer cell lines demonstrated IC_{50} values ranging from 9.9 μM to 32 μM, with the HEPG-2 as the most sensitive cell line [71].

Two metal complexes of Ni and Pt with 4[(2,2′:6′,2″-terpyridin)-4′-yl]-*N*,*N*-diethylaniline as the ligand were also developed. The Pt complex **26** was the most potent, with an IC_{50} of 1.5–40.3 μM against several cancer cell lines. Among them, T-24 cells (bladder carcinoma) were the most sensitive, with an IC_{50} of 1.52 μM. The G-4 stabilization was evaluated using the FRET-melting assay on *c-myc* G-4, showing a ΔT_m of +13.8 °C. In the TRAP assay, **26** displayed a telomere inhibition ratio of 60% at 1.5 μM, probably because of the interaction with the *c-myc* G-4-DNA. In addition, complex **26** was able to halt the cell cycle at S-phase, inducing apoptosis by disrupting mitochondrial function, at a concentration of 1.5 μM [72]. Furthermore, also in the field of metal complexes, the coumarin scaffold is a scaffold of choice for the development of active compounds. In this frame, Pt(II) complexes with 3-(2′-benzimidazolyl)-7-methoxycoumarin as the ligand were developed and tested for their anticancer activity and telomerase inhibition. The most potent was derivative **27**, which showed an IC_{50} of 0.5–45.1 μM against various cancer cells lines, of which the most sensitive was SK-OV-3/DPP cells (epithelial ovarian cancer). It displayed an inhibitory rate of 56% against telomerase at a concentration of 0.5 μM, binding to the *c-myc* promoter elements. Mechanistic studies revealed that **27** was able to arrest the cell cycle at the G2/M-phase and to induce apoptosis through the mitochondrial pathway [73]. In subsequent study by the same group, the anticancer properties of eight new Pt(II) complexes containing substituted 3-(2′-benzimidazolyl)coumarin as the ligand were investigated. The most potent compound **28** showed an inhibitory ratio of 52.3% at 1 μM concentration in SK-

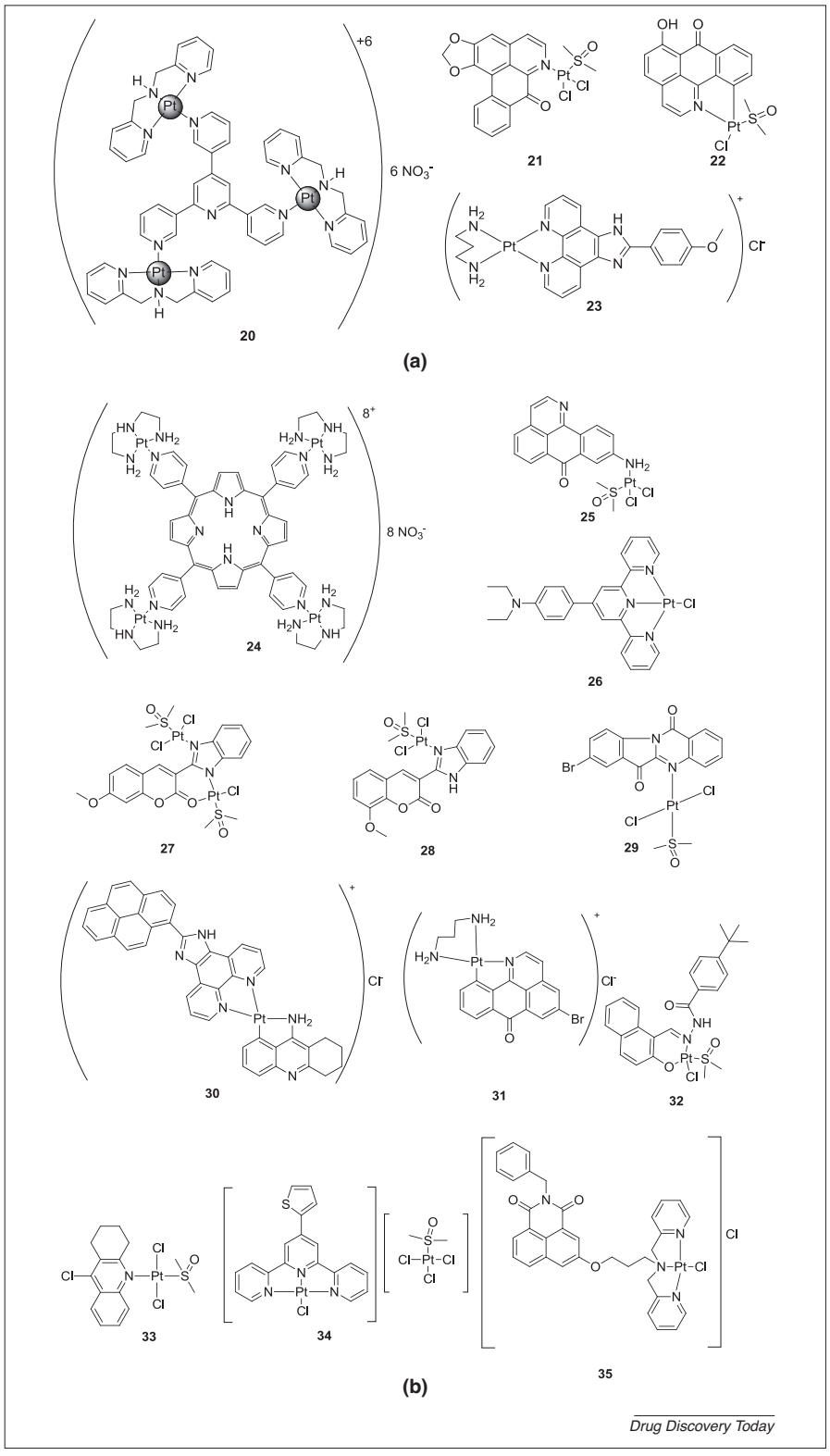

FIGURE 4

Platinum derivatives as G-quadruplex stabilizers: (a) compounds 20–23 and (b) compounds **24**–**35**.

OV-3/DDP cancer cells by targeting the *c-myc* promoter elements. A cytotoxicity assay showed IC_{50} values of 1.0–25.7 μM against HeLa, HEPG-2, SK-OV-3/DDP, and SK-OV-3 cell lines with, again, the most potent activity detected against the SK-OV-3/DDP cell line [74].

Quin and colleagues developed tryptanthrin-based Pt(II) complex **29** as a potential telomerase inhibitor. It showed cytotoxicity against different cancer cell lines, with IC_{50} values of 0.21–100.7 μM, with the highest activity against T-24 cells. The telomerase inhibitory ratio, analyzed by TRAP assay in T-24 cells at 0.2 μM,

was 56.4% targeting the *c-myc* promoter elements. A flow cytometry assay showed that this complex increased the percentage of T-24 cell in S-phase from 43.79% to 69.77%. In addition, it triggered apoptosis via the mitochondrial dysfunction pathway [75]. Recently, a tacrine-based Pt(II) complex, **30**, was developed that exhibited cytotoxicity against various cancer cell lines, with an IC_{50} of 0.13–5.01 μM, with the most potent activity against SK-OV-3/DDP cells. Its telomerase inhibition ratio, evaluated at 0.13 μM in SK-OV-3/DPP cells, was 52%, resulting from the direct targeting of the *c-myc* promoter elements. The proapoptotic effect of the compound resulted from dysfunction of mitochondria. From *in vivo* studies conducted in mice bearing HEPG-2 xenografts, **30** displayed a tumor growth inhibition ratio of 40.8%, lower than that of cisplatin, but, notably, with improved *in vivo* safety [76]. In another study, a 5-bromo-oxoisoporphine Pt(II) complex **31** was developed that exhibited a telomerase inhibition ratio of 50.8% in HEPG-2 cancer cells at 5 μM because of the interaction with the *c-myc* G-4. It demonstrated an IC_{50} range, in different cancer cell lines, of 5.06–18.09 μM, with the most potent activity on HEPG-2 strains. Complex **31** was also shown to induce apoptosis via the disruption of mitochondrial function [77].

A series of Pt(II) complexes derived from 2-hydroxy-1-naphthaldehyde benzoyl hydrazone were also developed. Compound **32**, with 4-*tert*-butyl-*N*-((2-hydroxynaphthalen-1-yl)methylene)benzohydrazide as the ligand, exhibited the highest potency, with an IC_{50} value of 4.38 μM, against the MGC80-3 cancer cell line. It inhibited telomerase activity with a ratio of 59% by targeting the *c-myc* promoter and downregulating the expression of human telomerase reverse transcriptase. It also caused cell cycle arrest at S-phase and triggered apoptosis by disrupting the mitochondrial pathway [78]. In another study, the Pt(II) complex **33** containing a 9-chloro-5,6,7,8-tetrahydroacridine moiety used as the ligand was developed. Cytotoxicity assays showed the most potent activity against HEPG-2 cancer cells with an IC_{50} value of 10.48 μM. The TRAP assay of **33** demonstrated an inhibitory rate of 31% at 10 μM in HEPG-2 cells. Complex **33** was able to interact with hTERT and *c-myc* G-4 and downregulate their expression [79].

In a recent study, the design, synthesis, and characterization of three new binuclear Pt(II) complexes, [Pt(tpbtpy)Cl][Pt(DMSO)Cl_3] (tpbtpy-Pt), [Pt(dthbtpy)Cl][Pt(DMSO)Cl_3].CH_3OH (dthbtpy-Pt), and [Pt(qlbtpy)Cl] [Pt(DMSO)Cl_3].CH_3OH (qlbtpy-Pt), with 4′-(3-thiophenecarboxaldehyde)-2,2′:6′,2″-terpyridine (tpbtpy), 4′-(3,5-bis (1,1-dimethylethyl)-2-hydroxy-benzaldehyde)-2,2′:6′,2″-terpyridine (dthbtpy), and 4′-(2-quinolinecarboxaldehyde)-2,2′:6′,2″-terpyridine (qlbtpy) as ligands, respectively, was reported. Their cytotoxicity was determined using a human non-small lung cancer cell line (NSCLC; NCI-H460 cells), exhibiting IC_{50} values of 0.35–12.09 μM. Compound **34** was the most potent, with an IC_{50} value of 0.35 ± 0.08 μM. Mechanistic studies indicated the **34** could induce apoptosis through mitochondrial dysfunction and inhibition of telomerase. *In vivo* studies using a NCI-H460 xenograft model (10.0 mg/kg every 2 days via intraperitoneal injection) revealed significant tumor reduction with a tumor growth inhibition rate (TGIR) of 70.1% on treatment with compound **34** [80]. In another report, two Pt(II) complexes with naphthalene imide moieties as ligands, were synthesized and characterized. Among these complexes, complex **35**, [Pt(Ligand)Cl]Cl, where ligand = 2-benzyl-5-(3-(*bis*(pyridine-2-ylmethyl)amino)propoxy)-1*H*-benzo(de)isoquinoline-1,3(2*H*)-dione, was the most potent, with high antitumor properties *in vitro* against NCI-H460 cells with an IC_{50} of 0.89 ± 0.25 μM. Complex **35** was also found to induce apoptosis by mitochondrial dysfunction and telomerase inhibition [81].

Ruthenium-based inhibitors

In work reported by Li and colleagues, two Ru-based complexes containing imidazole-[4,5-*f*][1,10]-phenanthroline and 2-phenylimidazo-[4,5-*f*][1,10]-phenanthroline as the ligands were generated. One of the two molecules (compound **36**, Fig. 5) exhibited interesting properties both on G-4 stabilization and telomerase inhibition. It was observed by FRET-melting assay that compound **36** could increase the ΔT_m value of F21T G-4 by 17.1 °C. TRAP assay results showed that **36** at a concentration range of 1–15 μM was able to completely inhibit telomerase activity. The PCR-stop assay confirmed the inhibitory activity of **36** against telo21 with an IC_{50} of 7.27 μM. Moreover, the *in vitro* activity of compound **36** was evaluated on various cell lines. The most potent action was seen against A549 human lung cancer cells, with an IC_{50} of 14.4 μM [82].

Two Ru polypyridyl complexes, **37** and **38** (Ru[$(bpy)_2$(pedppz)]$^{2+}$ and Ru[$(bpy)_2$(pemitatp)]$^{2+}$; (where bpy = 2′2-bipyridine, pdeppz = 10-(2-(piperidin-1-yl)ethoxy)dipyrido[3,2-*a*:2′,3′-*c*] phenazine and pemitatp = 5-methoxy-1-(2-(piperidin-1-yl)ethyl) isatino[1,2-*b*]-1,4,8,9-tetraazatriphenylene), with both G-4 stabilization and telomerase inhibition properties, were synthesized. A FRET-melting assay confirmed that both complexes increased the melting point of human telomeric sequences, at 3 μM concentration, by 11–15 °C. The TRAP assay showed potent inhibition of the telomerase activity for complexes **37** and **38** at 500 nM and 100 nM, respectively. The IC_{50} values, against HeLa cells, for the two molecules, were 38.8 μM and 39.7 μM, respectively [83]. The same research group identified complex **39**, namely ([Ru$(bpy)_2$(i-cip)]$^{2+}$ icip = 2-(indeno[2,1-*b*]chromen-6-yl)-1*H*-imidazo[4,5-*f*]-[1,10]phenanthroline), **40** [Ru$(bpy)_2$(pdppz)]$^{2+}$, (pdppz = phenanthro[4,5-*abc*]dipyrido[3,2-*h*:2′,3′-*j*]phenazine), and **41** [Ru$(bpy)_2$(-tactp)]$^{2+}$, (tactp = 4,5,9,18-tetraazachryseno[9,10-*b*]-triphenylene) as dual telomerase and topoisomerase inhibitors. FRET-melting assay results showed that complex **41** had the strongest interaction with G-4 DNA. At 1 μM concentration, it increased the melting point of F22 T DNA by +13 °C and by +18 °C at 3 μM concentration. A TRAP assay confirmed that complexes **40** and **41** were the most active, blocking completely telomerase activity at 100 nM concentration, whereas complex **39** had the same result at 500 nM concentration. Complexes **40** and **41** also showed inhibitory activity against topoisomerase I/II. Derivatives **40** and **41** also showed similar cytotoxicity to cisplatin against HeLa, HEPG-2, and A549 cell lines (21–23 μM, 21–27 μM, and 24–25 μM, respectively). All these complexes showed the ability to induce cell apoptosis [84].

A recent report described the discovery of water-soluble Ru(II) complexes with chiral 4-(2,3-dihydroxypropyl)formamide oxoaporphine as G-4 DNA stabilizers and telomerase inhibitors. The best compound, **42**, showed a broad spectrum of activity against many cancer cell lines, with a range of 7.4 (BEL-7404) to 69.2 μM (HL-7702). It also induced apoptotic cell death in a dose-dependent manner in BEL-7404 cell lines (HPV-related endocervical adenocarcinoma). The FRET-melting assay showed a ΔT_m value

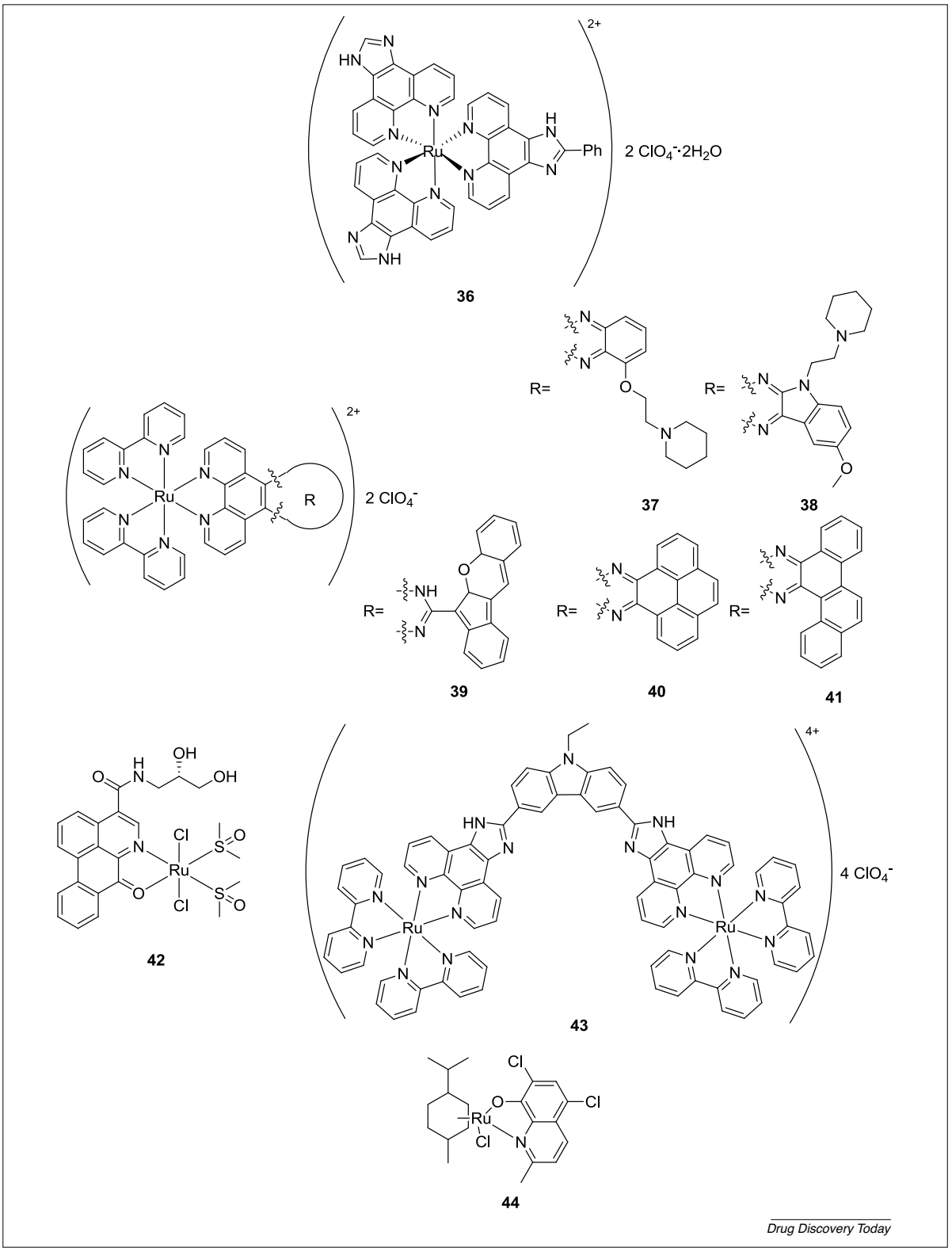

FIGURE 5

Ruthenium derivatives as G-quadruplex stabilizers (compounds **36**–**44**).

of +23 °C at a 2 μM concentration, indicating strong G-4-DNA selectivity. Compound **42** was observed to stabilize and down-regulate the *c-myc* and *hTERT* genes (analyzed in BEL-7404 cells). The TRAP assay demonstrated telomerase activity inhibition with an IC_{50} of 0.24 μM. An *in vivo* assay of **42** in a mouse model bearing BEL-7402 tumors showed dose-dependent inhibition (53% at 25 mg/kg of **42**) of tumor growth with greater potency and with higher safety than cisplatin [85]. In another work, binuclear Ru(II) complexes were synthesized; among them, complex **43** was found to be the most active, with a ΔT_m (in FRET-melting assay with F22T

G-4) of +20 °C, and complete telomerase inhibition at 200 nM (shown by TRAP assay). In addition, *in vitro* studies revealed that **43** exhibited cytotoxicity against the HeLa tumor cell line with an IC_{50} of 33.1 μM [86].

A series of novel 13 organometallic Ru(II)-arene complexes containing 5,7-dihalogenated-2-methyl-8-quinolinol as the ligand were synthesized, structurally characterized, and evaluated for their anticancer properties. Complex **44**, [Ru(η^6-p-cymene)Cl-(L1)], where L1 = 5,7-dichloro-2-methyl-8-quinolinol, was the most potent with an IC_{50} value of 2.00 ± 0.20 nM on HeLa cancer cells. Moreover, it induced apoptosis in HeLa cancer cells by telomerase inhibition and mitochondrial dysfunction, resulting in DNA damage as well as increased antimigration activity of HeLa cells. *In vivo* studies revealed that **44** exhibited high inhibitory activity against xenograft tumor growth of HeLa cells with TGIR of 58.5% [87].

Copper-based inhibitors

In a work by Majouga and colleagues, binuclear mixed-valence Cu(I, II) complexes containing substituted 2-alkylthio-5-arylmethylene-4*H*-imidazolin-4-ones as the ligands were developed. Compounds **45** and **46** (Fig. 6) showed potent *in vitro* anticancer activity against MCF-7, SiHa (human papillomavirus-related cervical squamous cell carcinoma), and HEK293 (human embryonic kidney) cell lines, with an IC_{50} range of 2–3 μM. Given the better solubility in biological media, compound **45** was chosen to be the lead compound and displayed telomerase inhibition with an IC_{50} of 6.5 μM in a TRAP assay. *In vivo* studies of breast adenocarcinoma growth in a C57BL/6 mouse model revealed that **45** caused tumor growth inhibition of 46.1% with an injection dose of 12 mg/kg [88]. Another work describes the development of the Cu (II)-based complex **47**, using 2-pyridine-thiosemicarbazone as a ligand. Using a cytotoxicity assay, **47** showed an IC_{50} of 0.8–1.68 μM in cancer cells, with the highest potency against MGC80-3 cells. It inhibited telomerase in a TRAP assay by 33.7%, at a concentration of 0.8 μM and reduced *c-myc* expression levels by 80% as well as inducing apoptosis [89]. A Cu(II) complex with dasatinib **48** was synthesized as a telomerase inhibitor and its anticancer activities were evaluated. The compound showed IC_{50} values of 4.0–13.0 μM in various cancer cell lines, with the most potent activity against HEPG-2 cells. It also inhibited telomerase activity (in a TRAP-PCR assay) with a ratio of 62.7% at a 4 μM concentration, targeting the *c-myc* promoter elements, and leading to an increased number of apoptotic cells [90]. In another study, a Cu-based complex using tryptanthrin as ligand (compound **49**) was analyzed. It demonstrated an IC_{50} of 4.0 μM in a cell growth inhibition study against the BEL-7402 cancer cell line and was shown to inhibit the activity of telomerase with a ratio of 48.9%, at the same concentration, by interacting with the *c-myc* promoter elements. Flow cytometry and western blot assays (analyzed in BEL-7402 cells) revealed that **49** could cause cell cycle arrest in S-phase, induce DNA damage, and trigger apoptosis, probably via the mitochondrial pathway [91].

Nickel-based inhibitors

Novel salen-based Ni(II) and Pd(II) complexes were developed to investigate their G-4 stabilization and telomerase inhibition. The most promising compound **50** (Fig. 7) demonstrated high binding affinity for G-4 DNA that was 420 times more selective than to double-stranded DNA. G-4 stabilization was also confirmed by CD melting assay (on preformed Hum_{21} G-4 DNA), resulting in an increase in the melting temperature of the

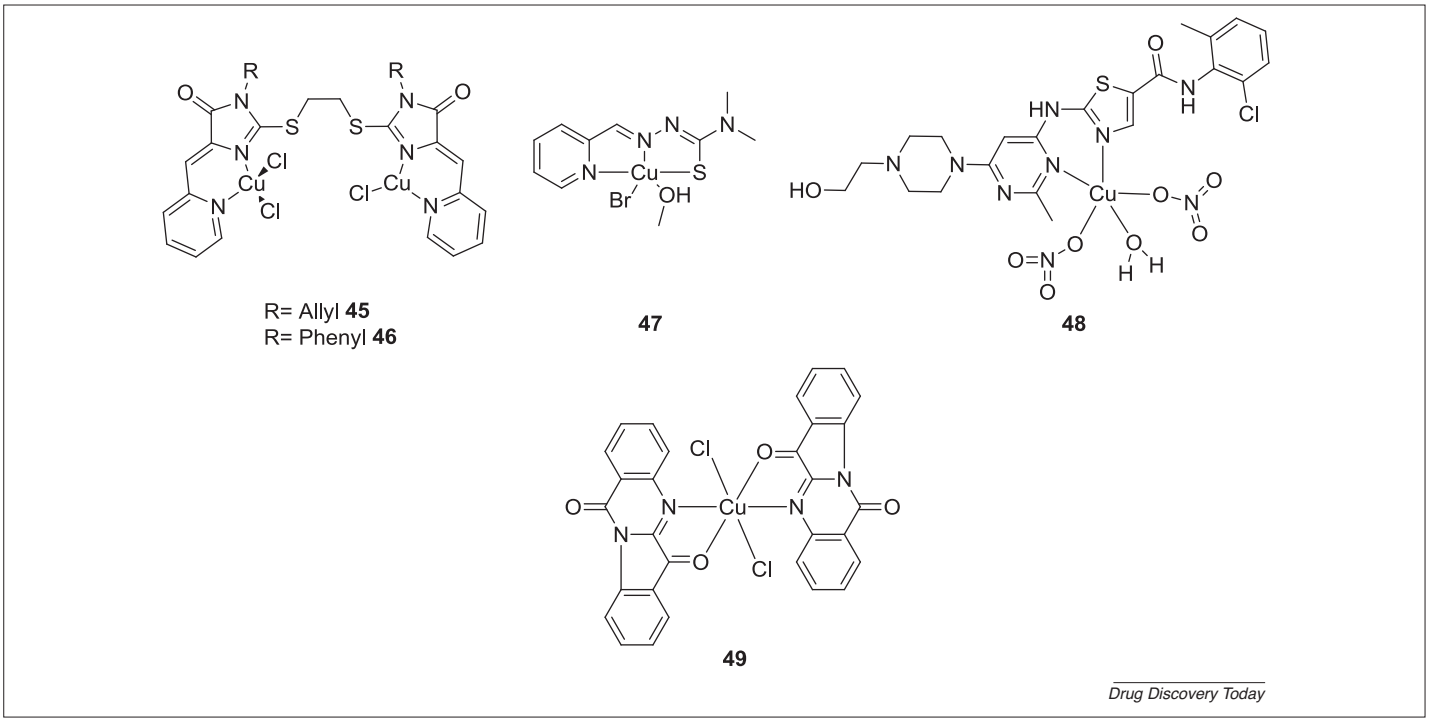

FIGURE 6

Copper derivatives as G-quadruplex stabilizers (compounds **45**–**49**).

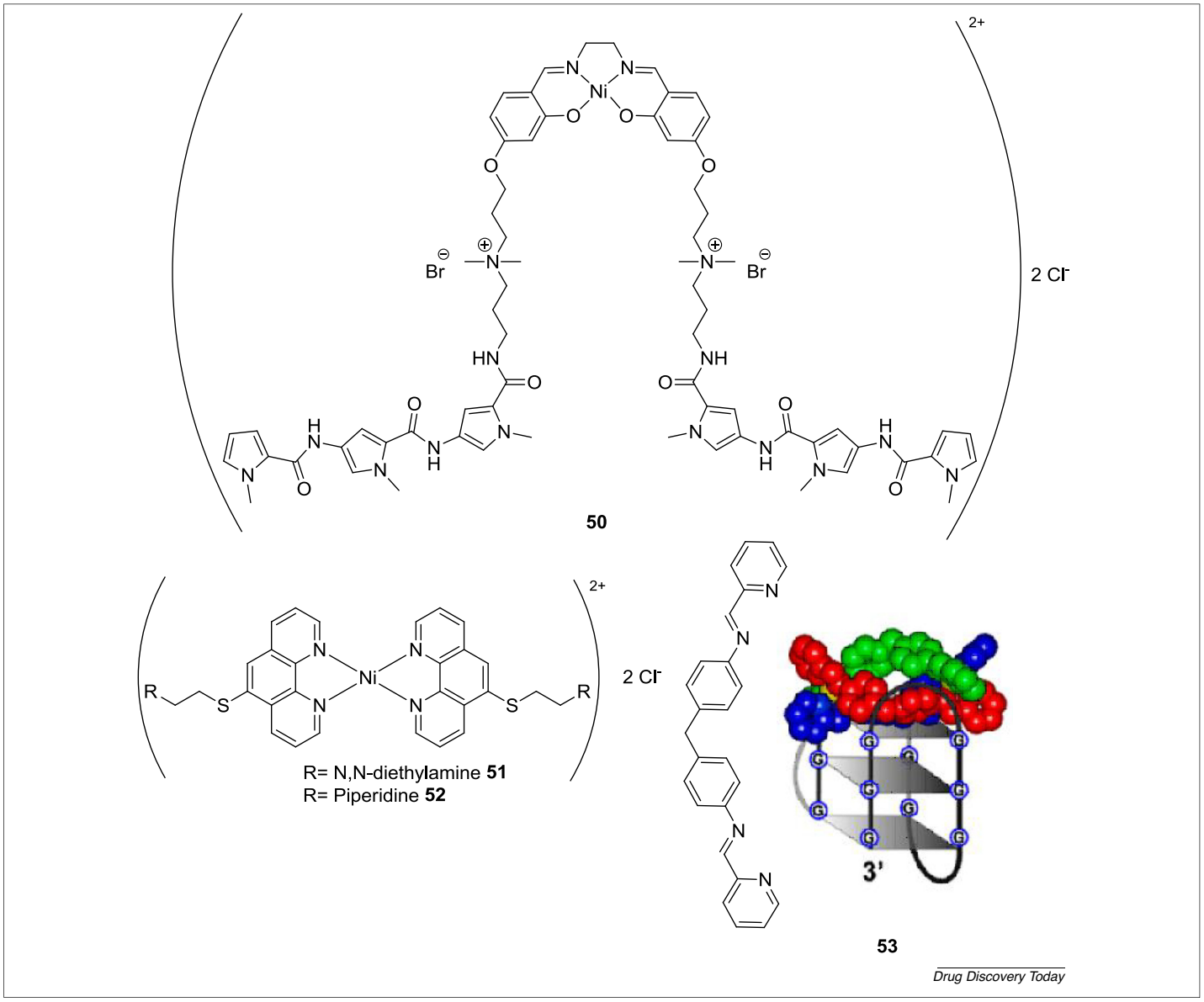

FIGURE 7

Nickel derivatives as G-quadruplex stabilizers (compounds **50**–**53**).

ligand–G-4 DNA complex of 16° when compared with free G-4 DNA. A submicromolar concentration (IC_{50} of 0.9 μM) of compound **50** led to complete inhibition of telomerase activity. A cell viability assay indicated that **50** caused cell death against the HEK 293T cancer cell strain with an IC_{50} value of 8 μM [92]. In another study, arylsulfanyl groups were used as side chains for 5-substituted 1,10-phenanthroline complexed with Ni(II), to evaluate their G-4 stabilization and telomerase inhibition. The best two compounds **51** and **52** showed in a FRET assay, a ΔT_m value of ~6–7 °C (with a complex/DNA ratio of 4:1) and a strong telomerase inhibition at a low micromolar concentration (12–6 μM) [93]. Ni-based complexes were also developed as a metal-supramolecular complex enantiomers with the general structure $[Ni_2L_3]^{4+}$(the structure of the ligand **53** and the interaction model of the complex with the G-4 DNA are described in Fig. 7). Complex **53** exhibited strong activity against breast cancer stem cells (CSCs) that are responsible for cancer recurrence, metastasis, and low life expectancy. In a TRAP G-4 assay, it displayed complete telomerase inhibition at 320 nM and an IC_{50} value of 73 nM. In living breast CSCs (MDA-MB231 and MCF-7 mammospheres), it exhibited telomerase inhibition with IC_{50} values of 1–2 μM. It was also involved in the loss of telomere-associated proteins (POT1 and TRF2) from telomeres, which led to a series of events, such as telomere DNA damage and apoptosis. Evaluation of the effects of this complex against CSCs in *in vivo* mouse models revealed complete inhibition of tumor formation compared with controls [94].

Other G-quadruplex stabilizers

Other transition metals complexes and their telomerase and anticancer activities have also been widely explored. Complex **54** [Cd (4-(2,3-dichlorophenyl)piperazine-1-carboxylic acid)·$2H_2O$] (Fig. 8) showed telomerase inhibition with an IC_{50} of 8.17 μM and inhibitory activity against the HEPG-2 cancer cell line of 6.5 μM [95]. In

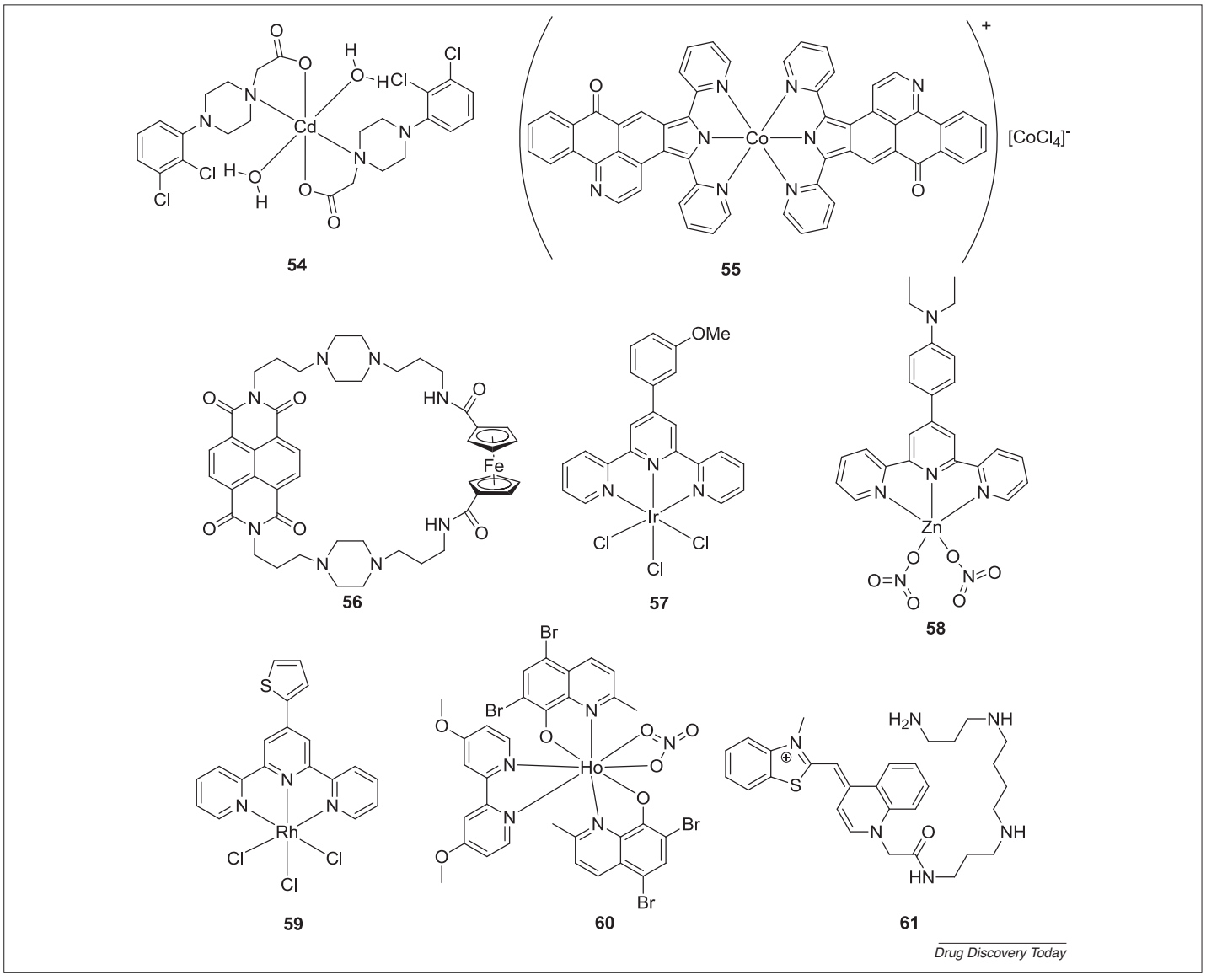

FIGURE 8

Other G-quadruplex stabilizers (compounds **54**–**61**).

another study, Co oxoisoaporphine was synthesized and its telomerase inhibitory properties were evaluated. Compound **55** was the most potent and was demonstrated to be a strong binder of G-4 DNA structures (the best affinity for *c-myc* G-4). A FRET-melting assay revealed that **55** increased the melting point of G-4 by 5–21 °C. The TRAP assay displayed a high inhibitory ratio against the activity of telomerase, reaching 73% inhibition at 1 μM concentration. A cytotoxicity assay revealed that it exerted the highest potency against SK-OV-3/DPP strain with an IC_{50} value of 1.0 μM. **55** was also seen to trigger cell senescence and apoptosis. An *in vivo* tumor growth inhibition assay performed using BEL-7402 and T-24 models showed an inhibitory rate of 62.1% at a concentration of 5 mg/kg, which is comparable with the efficacy of cisplatin, but with an improved safety profile [96]. Recently, a novel cyclic ferrocenyl-naphthalene diimide derivative, compound **56**, was synthesized as a G-4 stabilizer and a potential telomerase inhibitor. Spectrophotometric titrations showed that it exhibited higher affinity and selectivity for G-4 DNAs, up to 50-fold higher than for double-stranded DNA. This was confirmed also by a FRET-melting assay, where it obtained a ΔT_m of 5–18 °C in many G-4 DNAs (best result with *c-kit* and *c-myc*). The TRAP assay revealed that **56** exhibited telomerase inhibition with an IC_{50} of 0.4 μM [97].

Three Ir-based complexes, using 4′-(4-methoxyphenyl)-2,2′:6′2″-terpyridine as the ligand, were synthesized and subjected to biological evaluation as potential anticancer agents. The most potent compound **57** (Fig. 8) showed IC_{50} values of 3.2–27.8 μM in cytotoxicity assays, with the best activity against HEPG-2. The TRAP assay using HEPG-2 cells showed a telomerase inhibition ratio of 54.5% at 3.19 μM concentration achieved by targeting the *c-myc* promoter elements. **57** was also able to trigger apoptosis in the HEPG-2 cell via the mitochondrial pathway [98]. In another study, Zn(II) and Mn(II) complexes using 4-([2,2′:6′,2′-terpyridin]-4′-yl)-*N*,*N*-diethylaniline were developed. The compound had the best telomerase inhibitory ratio (61% at 1.3 μM concentration, TRAP assay) in a T-24 cell line was the Zn complex **58**. This inhibitory activity was strongly related to the interaction with

the *c-myc* G-4, which led to cell cycle arrest in S-phase. It demonstrated the best IC_{50} value against T-24 cancer cells at a concentration of 1.3 μM in a cytotoxicity assay [99]. Recently, a Rh(II) complex with 4′-(3-thiophenecarboxaldehyde)-2,2′:6′,2″-terpyridine as the ligand, namely compound **59**, was synthesized. In a cytotoxicity assay, it displayed the best potency against SK-OV-3/DDP cancer cells, with an IC_{50} value of 5.03 μM. Owing to the interaction with hTERT and *c-myc* G-4 DNA, it led to the down-regulation of these encoded proteins. In addition, it caused telomerase inhibition, which was evaluated by TRAP assay (5.0 μM of **59** in SK-OV-3/DDP cancer cells) with a ratio of 47.7% [100]. In another study, a novel series of lanthanides(III) complexes were developed as chelating ligands. *In vitro* cytotoxicity effects were determined on three cell lines: SK-OV-3/DDP, NCI-H460, and HeLa cancer cells. Complex **60** was the most potent, with an IC_{50} value of 1.00 ± 0.34 nM against the HeLa cells. This activity resulted from inhibition of proliferation by inhibiting telomerase and targeting of mitochondria to induce DNA damage-mediated apoptosis. *In vivo* studies performed on a HeLa mouse xenograft model showed a significant inhibition of tumor growth rate of 50.8% [101].

Besides several reports on metal-based G-4 stabilizers, Wang *et al*. recently synthesized a series of thiazole orange conjugates with different amino side chains as G-4 stabilizers. Their binding to telomeric G-4 DNA was investigated using biophysical methods, such as fluorescence-monitored thermal denaturation, circular dichroism, and fluorometric titration. Conjugate **61** (thiazole-spermine) exhibited the highest potency, with an IC_{50} value of 0.156 μM based on the TRAP assay, which was slightly better compared with the well-known G-4 ligand, BRACO-19 [102].

Blocking telomerase functioning: immunotherapy and vaccine-based strategies

The Nobel Prize in Physiology or Medicine 2018, awarded to James Allison and Tasuku Honjo for their work on how immune defense can be engaged for cancer treatment, highlights the potential of cancer immunotherapy. The telomerase active site in cancer cells is an attractive target to develop vaccines [103]. During proteasomal degradation of telomerase, the peptide fragments generated are expressed on the tumor surface as an antigen via the human leukocyte antigen (HLA) Class I pathway. The telomerase antigen epitopes are recognized by cytotoxic T lymphocytes (CTL), which then trigger an attack on cancer cells [104]. Telomerase-specific epitopes have the capacity to stimulate antigen-presenting cells (APC), leading to the induction of CD4+ and CD8+ cells or tumor destruction [105,106]. The absence of hTERT on the cellular membrane in benign cells, because of its predominant localization in the nucleus, provides an excellent opportunity to develop cancer-specific immunotherapeutics [103]. Currently, 18 immunotherapeutics and/or vaccines are in clinical trials, summarized in Table 2. Two main approaches of telomerase-based immunotherapy are: (i) direct activation of the immune system *in vivo;* and (ii) *ex vivo* activation and expansion of immune cells; both approaches have involved GV1001 and GRNVAC1, which are the most highly studied vaccines.

Direct activation of the immune system in vivo *(GV1001)*

GV1001 is a 16-amino acid (p611-EARPALLTSRLRFIPK-p626) containing the TERT-derived restricted peptide, and acts by direct activation of the immune system [26]. It is recognized by both major histocompatibility complex (MHC) Class I and Class II molecules, therefore eliciting CD8+ and CD4+ responses.

TABLE 2

Immunotherapeutics and vaccines targeting telomerase under clinical investigation[a]

Agent	NCT identifier	Phase	Condition	Status
CB-10-01 (transgenic lymphocyte immunization)	NCT00925314	2	Stage IIIB and IIIC skin melanoma	Unknown
GRNVAC1	NCT00510133	2	AML	Completed
GV1001	NCT01579188	3	Inoperable stage III NSCLC	Unknown
	NCT01342224	1	Locally advanced pancreatic adenocarcinoma	Completed
	NCT00509457	1	NSCLC	Completed
GV1001 (with gemcitabine hydrochloride and capecitabine)	NCT00425360	3	Pancreatic cancer	Completed
GV1001 in combination with temozolomide	NCT01247623	1/2	Malignant melanoma	Completed
GX301	NCT02293707	2	Prostate cancer	Active, not recruiting
hTERT mRNA dendritic cells	NCT01153113	1/2	Metastatic prostate cancer	Withdrawn
hTERT/survivin multi-peptide	NCT00573495	1	Breast neoplasm; breast cancer; ductal carcinoma	Completed
Telomerase [hTERT vaccine + pneumococcal conjugate vaccine (PCV)]	NCT00834665	1/2	Multiple myeloma	Unknown
Telomerase Peptide Plus GM-CSF (540-548)	NCT00069940	1	Brain and central nervous system tumors; gastrointestinal stromal tumor; sarcoma	Completed
Telomerase Peptide Plus GM-CSF (540-548)	NCT00079157	NA	Breast cancer	Unknown
emulsified in Montanide ISA-51	NCT00021164	2	Melanoma (skin); unspecified adult solid tumor	Completed
Transgenic lymphocyte immunization vaccine (TLI)	NCT00061035	1	Prostatic neoplasms	Completed
Tumor antigen-loaded autologous	NCT00197912	1/2	Advanced melanoma	Completed
dendritic cells	NCT00197860	1/2	Advanced renal cell carcinoma	Completed
UCPVax	NCT02818426	1/2	Metastatic NSCLC	Recruiting

[a] All data from https://clinicaltrials.gov/.

Currently, GV1001 is being screened in various cancers, including NSCLC, malignant melanoma, hepatocellular carcinoma, and pancreatic cancer [107]. Currently, no adverse effects have been observed after treatment with GV1001. Recent studies led to the observation that GV1001 penetrates the cell membrane, is localized in the cytoplasm, and reduces the levels of intracellular and surface heat shock proteins (HSPs) HSP70 and HSP90 [108]. In addition, GV1001 significantly reduces the levels of hypoxia-inducible factor α (HIF-1α) and vascular epithelial growth factor (VEGF) in cancer cells in hypoxic conditions. It also shows antioxidant and neuroprotective properties on neural stem cells by free radical scavenging and the modulation of survival and death signals [109].

Ex vivo *activation and expansion of immune cells (GRNVAC1)*

Treatment with GRNVAC1 involves an autologous vaccination approach in which mature autologous dendritic cells derived from patients are isolated and transfected *ex vivo* with mRNA (encoding hTERT and LAMP1) and administered via intradermal injections to patients [110]. This particular approach triggers TERT-specific CD8+ and CD4+ responses. Recently, a Phase II study of GRNVAC1 was completed in patients with AML. Results indicated that GNVAC1 administered in patients exhibited good tolerability and safety [111].

DNA vaccination is an effective emerging approach in cancer immunotherapy because it offers the flexibility to incorporate multiple genes [112]. INVAC-1, which is an optimized DNA plasmid encoding the inactivated form of hTERT, was recently designed to trigger cellular immunity against cancers. INVAC-1, when injected intradermally followed by electrogene transfer (EGT) in various mouse models, elicited a hTERT-specific cellular immune response, including higher levels of CD4 + Th1 effector and CD8+ memory T cells. Moreover, immunization with INVAC-1 in an HLA-A2 spontaneous and aggressive mouse sarcoma model slowed tumor growth and increased the survival rate by 50% of tumor-bearing mice, making it a promising vaccine candidate for the treatment of cancers. In addition, treatment with INVAC-1 was well tolerated and safe [113].

Telomerase in oral cancers

Oral and oropharyngeal cancers are the sixth most common forms of cancer globally, with an estimated 2.23 million deaths in 2008 [114]. Annually, 40 000 new cases of oral cancer emerge in the USA and 80,000 cases in India. Of oral cancers, 90% are oral squamous cell carcinomas (OSCC). Major risk factors include smoking and consumption of chewable tobacco. Oral carcinogenesis involves the transformation of normal epithelium to dysplastic lesions followed by progression of dysplastic lesions to invasive squamous carcinomas. Telomerase upregulation has been observed in 80% of OSCC, but not in normal oral cells, making telomerase a potential target for anticancer drugs [115]. Recent studies indicate the role of hTERT in promoting EMT. EMT is characterized by loss of cell to cell contact of epithelial cells and the ability to migrate, leading to invasiveness and metastasis, a key feature of various cancers. This is followed by activation of the Wnt/β-catenin pathway, stabilization of transcription factors Slug and Twist, and increased metastasis and invasion [116]. The role of telomerase in inducing chronic inflammation, which is considered a major risk factor in oral carcinogenesis, has been studied. Recent reports suggest that telomerase promotes the expression of the inflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) via activation of NF-κB to promote chronic inflammation [117]. However, the role of hTERT in tongue squamous cell carcinoma (TSCC) remains unknown. To investigate this, the CRISPR/ Cas9 gene-editing system was utilized to deplete hTERT in the SCC-15 (TSCC) cell line. A comparison with the SCC-15 control and knockout cells indicated that loss of hTERT suppressed cell proliferation and migration and significantly reduced tumorigenesis by mediating EMT. Mechanistic studies confirmed that hTERT knockout decreased signaling by NF-κB via a negative feedback system, highlighting the importance of targeting hTERT to improve the therapy for, and survival of, patients with TSCC [118]. The expression of hTERT in the dysplasia-carcinoma sequence in the oral cavity was examined using immunohistochemical methods. In addition, the effects of inflammatory cytokines on telomerase activity and migration of oral cancer cell lines (Ca9-22, HSC-3, and HSC-4) were studied. Immunoreactivity for hTERT was observed in squamous cell carcinoma and squamous intraepithelial neoplasia 3. Intriguingly, the activity of telomerase in Ca9-22 (gingival squamous cell carcinoma) cells increased on treatment with TNF-α and interferon (INF)-γ. However, its activity in HSC-4 cells was decreased by IL-1β. These results indicate that long-term inflammation increases telomerase activity, which in turn contributes to malignancy in oral mucosal epithelium [119]. Another study investigated whether the downregulation of hTERT induced an anti-invasive effect on an OSCC cell line, HSC3. Results showed that knockdown of hTERT inhibited the invasive activity of hTERT-overexpressing immortalized and HSC3 cells, as determined using a Transwell invasion assay and PCR for matrix metalloproteinases (MMP). Downregulation of hTERT also reduced MMP expression. An orthotopic xenograft model of nude mice was used for *in vivo* studies. These results support the targeting of hTERT to help develop innovative anticancer agents for oral cancers [120].

Major advances in the field of telomerase biology in oral cancers have recently been attained. Several methods have been developed to quantify telomerase activity in oral cancer cells, including an electrochemical telomerase assay (ECTA) and a PCR-based TRAP assay. ECTA uses ferrocenylnaphthalene diimide (FND) as a probe to detect telomerase activity in the lysate of the tumor tissue and neighboring cells of patients with oral cancer. The method involves immobilization of an electrochemical telomerase substrate (ETS) primer on an electrode. Telomerase was used to elongate the primer and FND bound to the product resulted in a current. In biopsy samples, the change in current was 30% more in patients and 20% less in healthy individuals. Positive rates of 85% and 90% were observed in cancerous and exfoliated tissues, respectively. Moreover, ECTA showed a 100% positive rate in early tumors <2 cm in size. The results showed that ECTA provided high hit rates for both cancerous and normal cells, indicating its suitability as a diagnostic tool for oral cancer [121]. In astudy aimed at quantifying telomerase activity in normal mucosal cells (20 specimens) and OSCC cells (from 45 patients), a PCR-based TRAP assay was used. Telomerase activity was detected in 89% of malignant and 5% of normal oral mucosa tissue, showing that the activation

of telomerase activity is frequent in OSCC and confirming its role as both a prognostic and diagnostic marker. No correlation was observed between telomerase activity levels and clinical stages, site of lesion, sex of the patients or history of adverse habits. However, increasing age of the patient had a positive correlation with the telomerase activity [122].

Concluding remarks

Over the past decade, there has been significant progress in exploiting the role of telomerase in carcinogenesis and ample evidence has been gathered to validate telomerase and telomere maintenance as promising targets for developing personalized cancer therapeutics. This is because of the selective expression of telomerase in cancer cells (85–90%) compared with normal human somatic cells, where it is almost absent. The new drug discovery opportunities supported by technological advances and biological insights that have emerged have provided impetus to the efforts to develop novel telomerase inhibitors. A deeper understanding of telomere biology and telomerase functions has led to the identification of novel diagnostic tools and effective potential anticancer agents [123]. Results from clinical trials have been encouraging because of the safety and good tolerability of telomerase inhibitors. Several small molecules, oligonucleotides, natural products, and immunotherapeutics are in various stages of advanced clinical studies for the treatment of a variety of malignancies. However, no telomerase-based cancer therapeutics have yet been approved by the US Food and Drug Administration (FDA) for clinical use because of the long time lag between administration of the drug and clinical response. Recent literature highlighting the advantages of targeting extratelomeric functions of telomerase-associated proteins could circumvent these delayed therapeutic effects and induce multifaceted antitumor effects. In this regard, both hTERT and dyskerin are considered attractive targets owing to their elevated expression in cancer cells compared with normal cells. Studies on dyskerin as a potential cancer target have paved the way for developing novel inhibitors. Continued advances in gene therapy and by exploiting the robust activity of the hTERT promoter in cancer cells as a viral vector are promising research avenues. In addition, immunotherapeutics targeting hTERT antigens are yielding impressive results and hold promise in the near future in cancer therapy. Small molecule-based telomerase inhibitors have not fared as well, but it might not take long for them to show a significant impact in clinical settings. A recently published 3D model of human telomerase based on electron microscopy images has guided scientists to a greater understanding of the telomerase structure [124]. Rational structure-based design of telomerase inhibitors will aim at increasing specificity, bioavailability, and inhibitory potential and could provide better outcomes compared with the currently available armamentarium of small-molecule inhibitors. Moreover, G-4 stabilizers hold immense potential as anticancer agents because of their ability to disrupt telomere structure by blocking the telomerase-binding proteins, TRF2 and POT1, and their high tolerance in the *in vivo* models. Therefore, targeting telomere and telomerase activity could be therapeutically beneficial in developing specific and potent anticancer drugs with selective toxicity to cancer cells. With regard to OSCC, no drugs are currently available to effectively treat and control the aggressiveness of either regional or distant metastases. Therefore, the development of diagnostic, prognostic, and therapeutic systems for OSCC would be a significant advancement, and could further help develop successful targeted therapies. hTERT could represent a novel therapeutic target for the treatment of OSCC tumors. A European Innovation Training Network (EU ITN)-Training in Cancer Mechanisms and Therapeutics (TRACT) has recently been set up in collaboration with academia and industry to investigate novel therapies for the treatment of OSCC.

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