Alternative Approaches to the Discovery and Development of Telomerase-Targeted Anticancer Drugs

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Abstract: Four different approaches have been reviewed herein: i) nucleoside analogs as mock agents of the reverse transcriptase (hTERT) catalytic site; ii) miscellaneous molecules with unknown mechanism(s) of action; iii) inhibitors of upstream processes of regulation of the hTERT subunit; iiii) immunotherapy against immunogenic hTERT- derived peptides.

INTRODUCTION

Telomerase is a ribonucleoprotein complex with RNA dependent DNA polymerase activity. In most eukaryotic cells, this specialized reverse transcriptase mediates the synthesis of guanine-rich DNA repeats onto telomeres, the ends of the chromosomes, using a template sequence within its integral RNA subunit [1]. In humans, telomeres consist of tandem repeats of the hexanucleotide sequence TTAGGG and are bound by a variety of proteins. DNA-protein complexes protect chromosomes from nuclease digestion, end-to-end fusion and other chromosomal rearrangement events [2].

Three components of human telomerase, human telomerase RNA component (hTR) [3], human telomerase protein 1 (hTEP1) [4, 5], and human telomerase reverse transcriptase (hTERT) [6, 7], have been identified recently. Most human cells do not express telomerase and lose telomeric DNA with each cell division. In contrast, the vast majority of human tumors exhibits strong telomerase activity [8] and maintains the length of their telomeres [9], suggesting that the activation of telomeres plays an important role in the development of human cancers. The telomerase catalytic subunit hTERT is the rate-limiting component of the complex, and its expression correlates best with telomerase activity [6, 7, 10]. The evidence that telomerase is activated in more than 85% of cancer cells, but not in normal cells, has led to study the usefulness of telomerase for cancer diagnostics and therapeutics.

A good telomerase inhibitor should meet the following criteria: 1) the inhibitor should reduce telomerase activity but initially, should not affect cell growth rates; 2) addition of the inhibitor should lead to progressive telomere shortening with each cell division; 3) addition of the inhibitor should eventually cause cells to die or to undergo growth arrest; 4) the time necessary to observe decreased proliferation should vary depending on initial telomere length; 5) chemically related molecules that do not inhibit telomerase activity should not cause decreased cell proliferation or telomere shortening [11].

Inhibitors of telomerase activity could target any one of several features of human telomerase. These include: hTERT active site; hTERT transcriptional active site; the 11-base RNA template; the anchor site where hTERT interacts with telomeric DNA; the extended telomere, possibly organized in a G-quadruplex structure; additional yet unknown proteins; antisense targeting of the mRNA for either hTR or hTERT.

The hTR template, and in general the nucleic acid portions of the telomerase machinery, as well as the G-quadruplexes of the nascent telomere repeats have been targeted by a number of strategies such as antisense DNA and RNA, protein-nucleic acids (PNA), ribozymes and small molecule stabilizers of G-tetraplex structures. These therapeutic approaches have been extensively discussed elsewhere in this issue. In this review, we will focus on the recent advances made in the inhibition of the hTERT catalytic component as an "alternative" yet sensible target of an anti-telomerase therapy. Four different approaches have been considered: nucleoside analogs as mock agents of hTERT catalytic site; small miscellaneous molecules which are selected based on the effectiveness of telomerase inhibition rather than a knowledge of their mechanism of action; inhibitors of an upstream process of transcription and/or regulation of the hTERT subunit; immunotherapy against immunogenic hTERT derived peptides.

REVERSE TRANSCRIPTASE INHIBITORS

Telomerase is a specialized DNA polymerase ribonucleoprotein. Functionally, it belongs to a class of enzymes known as reverse transcriptases that use RNA as a template for producing DNA. The crystal structure of telomerase is still unknown. However, the catalytic subunit possesses typical motifs found in reverse transcriptases [6, 12] and mutations made at conserved residues within motifs common to hTERT and various reverse transcriptases have been found to abolish or reduce telomerase activity [13]. Consequently, reverse transcriptase inhibitors such as those used in anti-HIV chemotherapy are good candidates to

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inhibit telomerase. One major class of reverse transcriptase inhibitors, nucleoside analogs, is currently under investigation against telomerase catalytic subunit.

Nucleoside analogs are pyrimidine or purine analogs that differ from natural nucleosides for chemical modifications to their ring residues. The modified nucleosides are still recognized by the reverse transcriptase enzyme, but the chemical modified key moieties inhibit the subsequent step of elongation.

Nucleoside analogs are normally phosphorylated to the triphosphate form by host cell enzymes and incorporated into the newly synthesized DNA chain where they inhibit further elongation. The ability of nucleoside analogs to efficiently and specifically inhibit reverse transcriptase lie in the preferential recognition of the drug by the enzyme with reference to the endogenous nucleoside triphosphate. Furthermore, modified nucleosides also may be processed by cellular DNA polymerases, and the success of an anti-reverse transcriptase drug consists in its ability to specifically mock the target enzyme while being recognized and discarded as non-natural substrate by the related enzymes. This rationale applies to the use of telomerase nucleoside analogs that need to be selective for reverse transcriptase while poorly affecting all other cellular polymerases.

In this account, identification of nucleotide inhibitors that are selective for telomerase may take advantage of

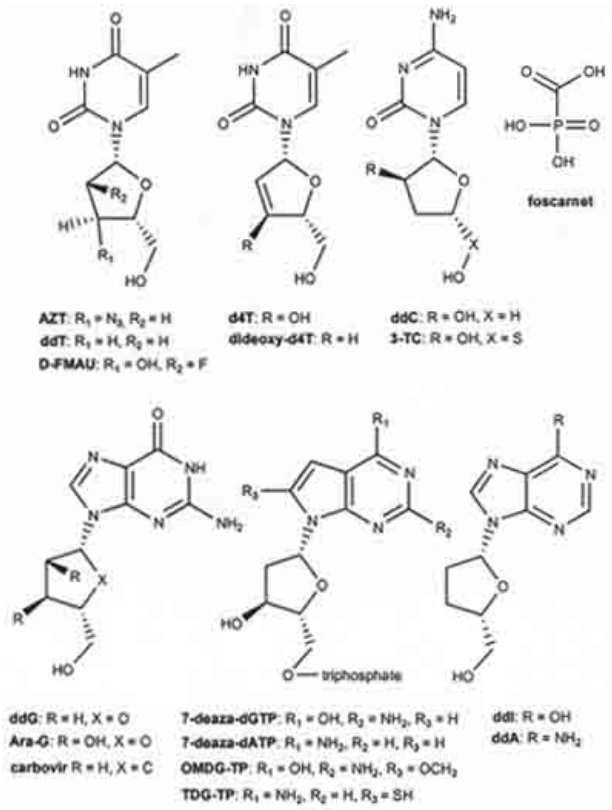

Fig. (1). Reverse transcriptase (hTERT) inhibitors. Chemical structures of nucleoside analog inhibitors of the reverse transcriptase catalytic subunit of telomerase.

reverse transcriptase inhibitors synthesized during programs aimed to investigate the inhibition of other polymerases. Indeed most reverse transcriptase inhibitors used in the treatment of HIV have been tested for telomerase inhibition.

Nucleotide analog inhibitors are grouped in figure **1**.

3'-azido-3'deoxythymidine (AZT)

3'-azido-3'deoxythymidine (AZT) and AZT triphosphate (AZT TP) have been most extensively studied. In chronological order, AZT has first been shown to inhibit growth and telomerase activity of Tetrahymena and lead to progressive shortening of telomeres [14] both in Tetrahymena and in B (cell line JY616) and T (cell line Jurkat E6-1) cell cultures [15]. On B and T cell lines AZT-TP decreased telomerase activity *in vitro* [15]. AZT was next found to decrease mammalian cell growth and induce senescence like processes in cultures of immortal mouse fibroblastes with changes in cellular morphology. The process was reversible after removal of the RT inhibitor [16]. When exposed to long-term treatment to AZT, both HeLa [17] and HEC-1 tumor cells [18] reported shortening in telomeric sequences. In this case no evidence of senescence could be detected. AZT was also proved to inhibit breast cancer cell growth [19] and to have high inhibitory activity on a partially purified telomerase from the blast cells of patients with acute myelogenous leukemia [20]. AZT blocked telomerase function and induced senescence, partial differentiation or crisis in various cells among which are 3T3 Swiss, NIH 3T3 and immortal spontaneously transformed mouse fibroblasts, L6 rat myoblasts and the human tumor cell lines U-937 and MeWo. In human tumor cells, AZT mainly induced crisis [21].

When used for short periods of time at high concentrations (IC_{50} values above 200 μM), AZT showed very low cytotoxic effect against both normal and different tumor cell lines [22]. More recently the effects of chronic *in vitro* AZT exposure were investigated on F3II mouse mammary carcinoma cells. AZT-treated tumor cells showed a reduced tumorigenicity in syngeneic BALB/c mice. Tumor incidence was reduced and survival was prolonged in animals inoculated with AZT-treated cells when comparing with control counterparts. The number and size of spontaneous metastases were also decreased in animals inoculated with AZT-treated cells. Morphological and biochemical evidence of senescence and induction of programmed cell death was also found [23].

Dideoxyguanosine (ddG)

Dideoxyguanosine (ddG) has been shown to cause reproducible, progressive telomere shortening in human B and T cell cultures over several weeks of passaging, after which the telomeres stabilized and remained short. However, the prolonged passaging in ddG caused no observable effects on cell population doubling rates or morphology [15]. Telomerase activity was inhibited *in vitro* by ddGTP.

The inhibitor ddGTP has been reported to be more potent than AZT against telomerase function in acute myelogenous

leukemia blast cells [20], while it reduced telomerase activity by 47 % compared to 97% with AZT, on extracts of colorectal cells [24].

Other Nucleoside Analogs

More nucleosides analogs have been tested for telomerase inhibition, but the results have thus far been inconsistent. Didehydrothymidine (d4T), but not dideoxyinosine (ddI), inhibited the growth of Tetrahymena and led to shortening of telomeres [14], however, prolonged passaging of B or T cell cultures in (d4T), (ddI), arabinofuranyl-guanosine (Ara-G), dideoxyadenosine (ddA), or phosphonoformic acid (foscarnet) did not cause reproducible telomere shortening or decreased cell growth rates or viabilities. Furthermore, combining foscarnet, and/or arabinofuranyl-guanosine with ddG did not significantly augment the effects of ddG alone [15]. Other papers claimed that carbovir, d4T, dideoxythymidine (ddT) and 2',3'-dideoxy 2',3'didehydrothymidine (dideoxy-d4T) retained inhibitory effects on telomerase, but not ddI or dideoxycytidine (ddCTP) [16, 20, 25, 24]. The inactivity of ddCTP was explained as a result of the sequence synthesized by telomerase: the TTAGGG repeat does not contain deoxycytidine; hence, its analog cannot influence the function of the enzyme.

L-enantiomer Nucleoside Analogs

In the case of HIV-1 reverse transcriptase, it has been reported that the enzyme binds and incorporates the L-enantiomer of the natural substrate dTTP *in vitro* [26, 27], thus HIV-1 RT is considered as a poorly enantioselective enzyme. Indeed, the L-nucleoside analog (-)-2'-deoxy-3'-thiacytidine (3-TC) has been approved for clinical use against HIV-1 [28, 29]. However the L-enantiomers of carbocyclic dGTP [30] and 2'-fluoro-5-methylarabinofuranosyluracil 5'-triphosphate (D-FMAU-TP) [20] were far less inhibitory against human telomerase, suggesting a stereoselectivity of telomerase for nucleotide substrates. Yamaguchi *et al.* showed that among the four L-dNTPs, L-dTTP and L-dGTP inhibited telomerase activity and the others showed slight or no inhibitory effect. However, the K(i) values of L-dTTP and L-dGTP suggested that the active site of telomerase was not able to strictly discriminate the chirality of dNTPs, although it was more discriminatory than HIV-1 RT [31, 32].

7-deaza Nucleoside Analogs

Inhibitory activity of telomerase has been reported for two 7-deaza-nucleotide triphosphate derivatives, 7-deaza-2'-deoxyguanosine-5'-triphosphate (7-deaza-dGTP) and 7-deaza-2'-deoxyadenosine-5'-triphosphate (7-deaza-dATP), with IC_{50} comparable to those of their deoxynucleotide homolog, dGTP and dATP. Additional studies showed that both 7-deaza-dGTP and 7-deaza-dATP were also incorporated into telomeric DNA by telomerase and, as a result, the telomeric ladder was prematurely shortened [33]. Two new telomerase-inhibiting nucleotides were more recently reported: 6-methoxy-7-deaza-2'-deoxyguanosine 5'-triphosphate (OMDG-TP) and 6-thio-7-deaza-2'-deoxyguanosine 5'-triphosphate (TDG-TP). In particular, TDG-TP behaved as a very potent inhibitor of human telomerase with an IC_{50} of 60 nM. When TDG-TP was the only available guanosine substrate, telomerase became non-processive, synthesizing short products that appeared to contain only one to three TDG residues. Similarly, the less potent telomerase inhibitor OMDG-TP gave rise to short telomerase products. However, TDG-TP and, to a lesser extent, OMDG-TP, were non-telomerase specific since they could serve as a substrate for both templated (Klenow exo) and non-templated (terminal transferase) DNA polymerases [34]. The suggested mechanism for 7-deaza-derivatives is that their incorporation into telomeric DNA would impair or prevent the formation of secondary telomeric DNA tetraplex structures: in fact N-7 is required for interguanine hydrogen bonding within a planar G4-tetrad array and tetraplex formation is proposed to facilitate translocation. Hence, hindering translocation would result in shortened telomerase products. Alternative mechanisms of destabilization of DNA/RNA duplex and/or competitive substrate inhibition may also be considered.

To date, no nucleoside-analog inhibitor has been found to possess adequate selectivity for telomerase alone, all being active to appreciable extents on different reverse transcriptases and polymerases. The recent cloning of hTERT catalytic subunit has not led so far to mass screening of these derivatives and probably hTERT high-resolution structural information is needed to facilitate development of nucleoside analogs.

MISCELLANEOUS INHIBITORS

The complexity of the telomere-telomerase complex makes it possible to consider random and high-throughput screening programs as a rational approach to the discovery and development of highly selective and potent non-nucleoside inhibitors of the telomerase function.

Natural Compounds

Many natural compounds have been successfully tested as selective anti-telomerase anticancer drugs, even though the molecular details of the inhibition are not known yet (chemical structures are shown in figure **2**). Potent anticancer effects due to tea were initially observed in animal *in vivo* studies and in human epidemiological observations. Naasani *et al.* demonstrated that epigallocatechin gallate (EGCG), a major tea catechin, strongly and directly inhibited telomerase in a cell-free system (cell extract) as well as in living cells. Continued growth of two human cancer cell lines, U937 monoblastoid leukemia cells and HT29 colon adenocarcinoma cells, in the presence of nontoxic concentrations of EGCG showed life span limitations accompanied by telomere shortening, chromosomal abnormalities, and senescence [35].

Alterperylenol, a fungus metabolite, has been shown to selectively inhibit telomerase activity (IC_{50} = 30 μM), without affecting the activity of viral reverse transcriptase at 1 mM [36].

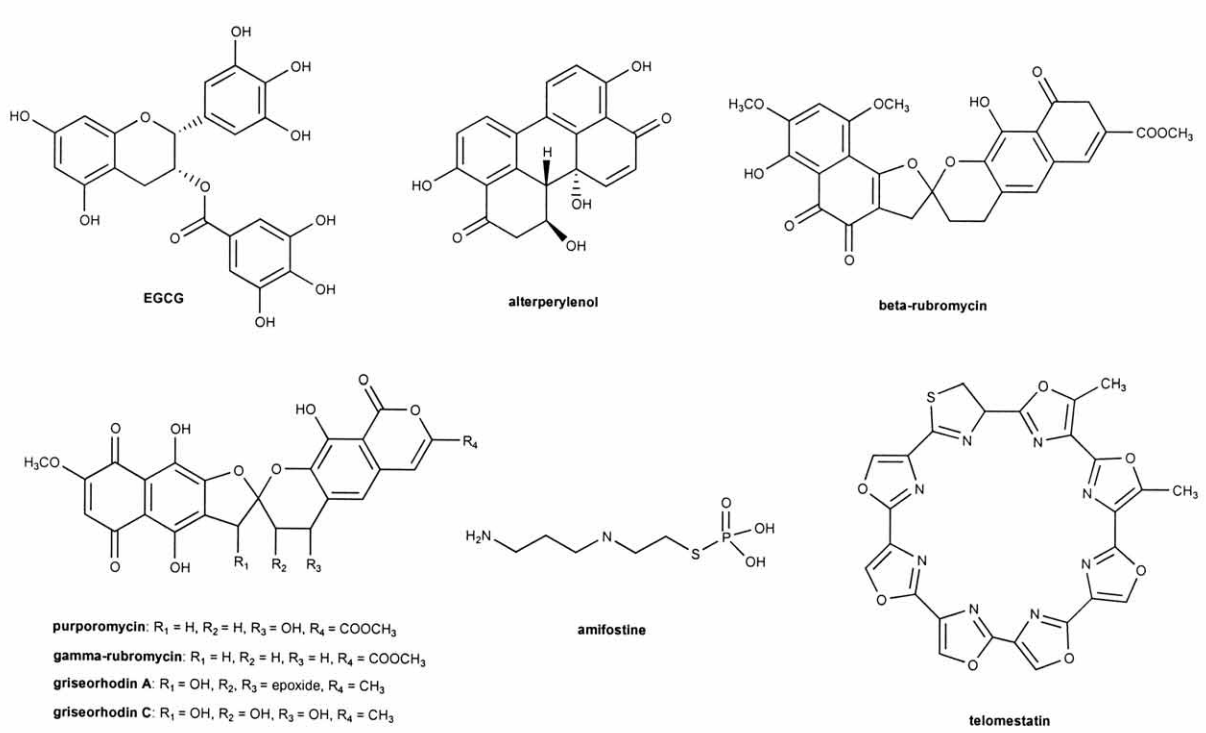

Fig. (2). Miscellaneous natural molecules. Chemical structures of natural molecules with unknown mechanism(s) of inhibition of telomerase activity.

Interleukin (IL) 4 and amifostine, two natural non-cytotoxic agents, were also shown to reduce telomerase activity within leukemia cells *in vivo* in patients [37].

More recently a screening program to identify telomerase inhibitors from fungus fermentation sources led to the discovery of two compounds, CRM646-A and thielavin B, which inhibited telomerase activity at doses of 3.2 and 32 μM, respectively. However these compounds were not selective against telomerase since they were able to inhibit viral reverse transcriptase at almost the same dose levels used to inhibit telomerase [38].

Other natural compounds have been found to inhibit telomerase as a result of wide range screening of Streptomyces metabolites. Among them rubromycins and their analog, a class of quinone antibiotics that possesses benzofuran and benzodipyran rings to form a spiroketal system, were very effective. Beta- and gamma-rubromycins and purpuromycin were the most potent telomerase inhibitors, (IC_{50} of about 3 μM), and griseorhodins A and C also showed comparable potencies for the inhibition (IC_{50} = 6-12 μM). The spiroketal system of beta-rubromycin was essential for telomerase inhibition. Beta-rubromycin was also active against retroviral reverse transcriptases but had virtually no effect on other DNA/RNA-modifying enzymes including DNA and RNA polymerases, deoxyribonuclease, and topoisomerase [39]. Telomestatin also came from the screening of Streptomyces metabolites: it consists of 7 oxazole rings and 1 thiazoline ring covalently bound to form a macrocyclic planar molecule. Telomestatin specifically and potently inhibited telomerase activity (IC_{50} = 5 nM), whereas it did not show activity against DNA polymerases, such as Taq polymerase, and exhibited only weak inhibition on different reverse transcriptases, such as HIV-1 and MMLV (Moloney Murine Leukemia Virus) RTs. If confirmed, these data would prove telomestatin to be one of the most potent and specific telomerase inhibitor reported to date [40].

Chemically Synthesized Compounds

High-throughput screening of a number of chemical libraries has led to the identification of several non-natural molecules, which have been proved to be good inhibitors of telomerase activity. The systematic screening of two different libraries of 125,000 and 16,000 compounds by two research groups resulted in the identification of a set of isothiazolone–containing telomerase inhibitors. In one case, the most potent drug showed submicromolar IC_{50} values [41], in the other case the most potent inhibitor was 2-[3-(trifluoromethyl)phenyl]isothiazolin-3-one (TMPI), which inhibited telomerase activity at 1.0 μM in extracts of cultured human cells [42] (fig. **3**). Furthermore, TMPI did not inhibit eukaryotic DNA polymerase alpha, beta, or human immunodeficiency virus reverse transcriptase (HIV RT), indicating that inhibition by TMPI was highly selective for telomerase. TMPI was suggested to act at the cysteine residues of the enzyme.

COMPARE analysis on the database of a disease-oriented screening program (DOS) was exploited to identify the alkaloid berberine (fig. **3**) as a moderate telomerase

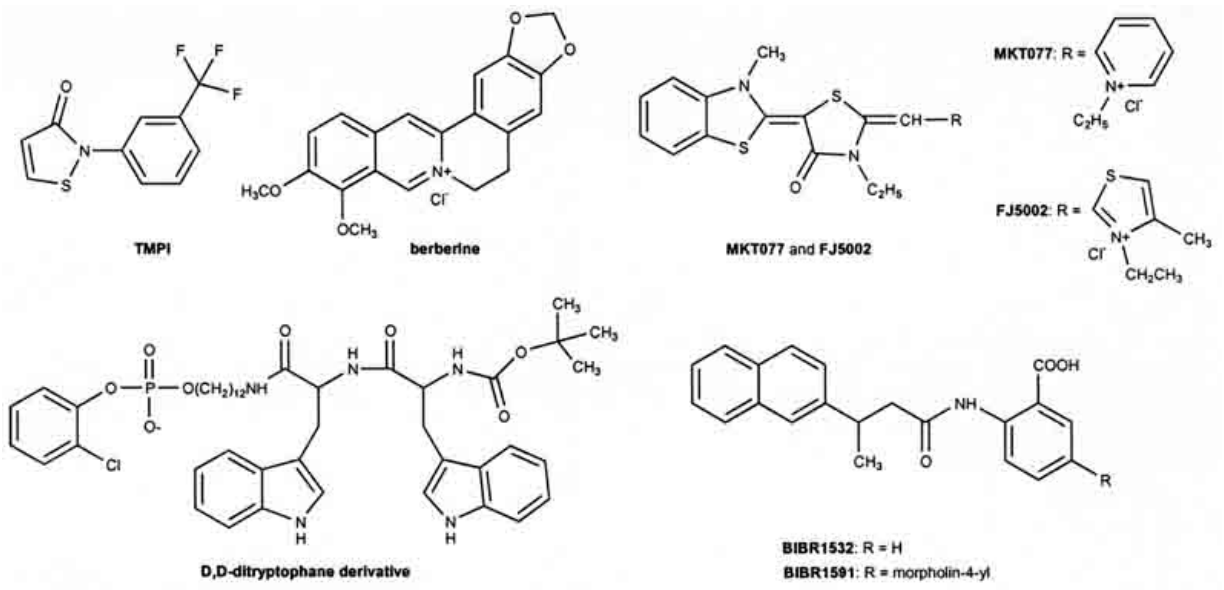

Fig. (3). Miscellaneous chemically synthesized molecules. Chemical structures of synthetic organic compounds with unknown mechanism of action against telomerase activity.

inhibitor with approximately 35 μM IC_{50}. This alkaloid was subsequently used as a seed compound to identify other berberine-like compounds. Among these compounds, MKT077 (fig. **3**), a rhodacyanine derivative currently under Phase I clinical trials, showed a potent inhibitory effect in the low μM range. With MKT077 as an upgraded seed for a new round of COMPARE analysis, rhodacyanine FJ5002 (fig. **3**), a close derivative of MKT077, was identified as the most potent telomerase inhibitor with 2 μM IC_{50}. Subacute concentrations of FJ5002 on a long-term cultivation of U937, a human leukemia cell line, resulted in population-doubling dependent changes characterized by progressive telomere erosion, increased chromosome abnormalities, and senescence/crisis-like features indicating that FJ5002 is a promising telomerase inhibitor [43].

Sasaki *et al.* recently constructed a library of solid phase synthetic compounds based on three structural units: i) a phosphate with a hydrophobic group, ii) a bisindole unit, iii) a long alkyl spacer between them. Among the library components, a D,D-ditryptophane derivative (fig. **3**) has been identified as a new potent telomerase inhibitor with IC_{50} values of 0.3 μM. Based on a structure-activity relationship (SAR) the authors proposed a model for a hypothetical binding site of dipeptide-type inhibitors on telomerase catalytic subunit. The inhibitor binding site would consist of a phosphate binding site, a hydrophobic pocket, a hydrophobic site, and an indole, an aromatic and a Boc site [44].

Systematic SAR allowed the identification of two compounds, BIBR1591 and BIBR1532 (fig. **3**). They selectively inhibited telomerase *in vitro* and *in vivo* leading to progressive telomere shortening, with no acute cytotoxicity, but a proliferation arrest after a characteristic lag period with hallmarks of senescence, including morphological, mitotic and chromosomal aberrations and altered patterns of gene expression. Telomerase inhibition and telomere shortening also resulted in a marked reduction of the tumorigenic potential of drug-treated tumor cells in a mouse xenograft model. This model was also used to demonstrate *in vivo* efficacy with no adverse side-effects and uncomplicated oral administration of the inhibitor. Furthermore, these compounds did not show activity against DNA or RNA polymerases, including HIV-1 reverse transcriptase [45]. The mode of BIBR1532 action has been characterized: the drug inhibits the native and recombinant human telomerase, comprising the hTERT and hTR components with similar potency, primarily by interfering with the processivity of the enzyme. Enzyme-kinetics experiments showed that BIBR1532 is a mixed-type noncompetitive inhibitor and suggested a drug-binding site distinct from the sites for deoxyribonucleotides and the DNA primer, respectively. Thus, BIBR1532 is the representative of a novel class of telomerase inhibitors, with mechanistic similarities to non-nucleosidic inhibitors of HIV-1 reverse transcriptase [46].

Anticancer Drugs

The mechanism of action of many anticancer drugs is not yet fully understood. Several studies investigated the effects of established anticancer drugs on telomerase activity in cell cultures in the attempt to determine if telomerase inhibition is a mechanistic component of drug efficacy.

Chemical structures of anticancer drugs used as telomerase inhibitors are shown in fig. **4**.

Tamoxifen is a hormonal agent (antiestrogen) touted as the endocrinal treatment of choice for all stages of breast carcinoma. One mechanism of action of tamoxifen is to compete with estrogen by binding to the estrogen receptors to inhibit cancer cell growth. The effects of tamoxifen on telomerase activity were tested both in estrogen receptor positive (MCF-7) and estrogen receptor negative cell lines (MDA-MB-231). Indeed, tamoxifen treatment (10 nM)

induced lower total cell counts and lower telomerase activity levels than in control cells and changes in the expression of individual telomerase components correlated with telomerase activity, while estrogen receptor status did not correlate with telomerase activity [47].

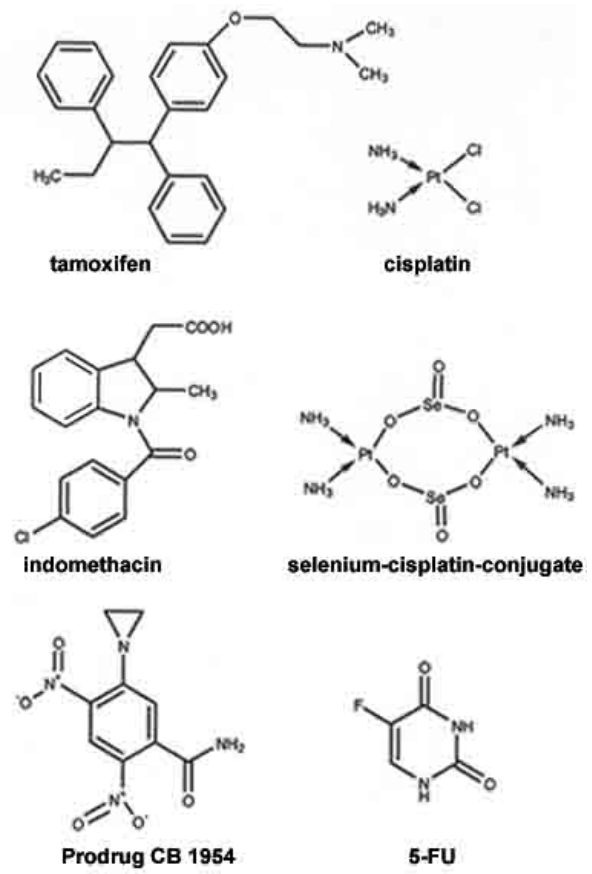

Fig. (4). Anticancer drugs. Small molecules, already used as anticancer drugs, with miscellaneous mechanism(s) of inhibition of telomerase activity.

Cycloogygenase inhibitors (indomethacin, mobic, sulindac sulfone, suramin) were demonstrated to retard tumor growth both in murine tumors and in human tumor cells by inhibition of telomerase activity in addition to previously recognized mechanisms as induction of apoptosis, inhibition of cell proliferation, influence on the expression of growth factors around growing tumors and attenuation of neoangiogenesis [48].

The antineoplastic agents cisplatin, VP-16, vincristine and gamma-irradiation were also tested for telomerase activity through a comprehensive set of experiments and telomerase activity was down-regulated by these agents in lymphoma cells; however, expression of hTERT did not correlate with telomerase activity [49].

Conversely, TE-9 cells exposed to 5-fluorouracil (5-FU) showed a diminished telomerase activity, preceded by a time dependent decrease in the mRNA expression of hTERT catalytic subunit.

In earlier work, cisplatin was tested for its ability to inhibit telomerase function with the rational that telomeric tandem repeats as well as the human telomerase RNA component (hTR) and its gene are guanosine-rich and cisplatin is known to bind preferentially and cross-link G-tract regions of duplex DNA with sequence specificity G-Pt-G. Cisplatin was found to reduce telomerase activity in a specific and concentration-dependent manner in human testicular tumor cells, whilst doxorubicin, bleomycin, methotrexate, melphalan and transplatin had no effect. [50]. Cisplatin induced inhibition of telomerase in oral squamous cell carcinoma and cells with high telomerase expression tended to be resistant to the drug [51]. Very recent work showed that a novel potential anticancer compound, a conjugate of selenite with diammineplatinum $[(NH_3)_2Pt(SeO_3)]_2$, induced, in a concentration-dependent manner, both damage to DNA and reduction of telomerase activity in endometrial cancer cells derived from tumor samples. Sodium ascorbate at 10 and 50 μM reduced the extent of the DNA damage evoked by the drug, but telomerase inhibition was independent of sodium ascorbate. Therefore, mutagenic effects of the conjugate could be reduced by the well-recognized antimutagen, sodium ascorbate, but the conjugate could still retain ability to affect neoplastic transformation. The results indicated that $[(NH_3)_2Pt(SeO_3)]_2$ specifically inhibits telomerase activity in endometrial cancer cells [52].

Gene-directed Enzyme Pro-drug Therapy (GDEPT)

The feasibility of gene-directed enzyme pro-drug therapy (GDEPT) against telomerase activity has been demonstrated recently.

The transcriptional regulatory sequences from the hTERT and hTR genes were used to regulate expression of the bacterial nitroreductase enzyme in combination with the pro-drug CB1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide) (fig. **4**) in a suicide gene therapy strategy. Placing the nitroreductase gene under the control of the telomerase gene promoters sensitized cancer cells in tissue culture to the pro-drug CB1954 and promoter activity was predictive of sensitization to the pro-drug (2-20-fold sensitization), with cell death restricted to lines exhibiting high levels of promoter activity. The *in vivo* relevance of these data was tested using two xenograft models (C33a and GLC4 cells). Significant tumor reduction was seen with both telomerase promoters, and the promoter-specific patterns of sensitization observed in tissue culture were retained in xenograft models. Thus, telomerase-specific suicide gene therapy vectors expressing bacterial nitroreductase sensitize human cancer cells to the pro-drug CB1954 [53].

HTERT-EXPRESSION REGULATORS

To date, three major subunits comprising the human telomerase complex have been identified. The RNA component of telomerase (hTR) provides the template for telomere repeat synthesis [3]. Telomerase-associated protein 1 (TP1) is a telomerase component with the still unclear function [5]. The most important component responsible for

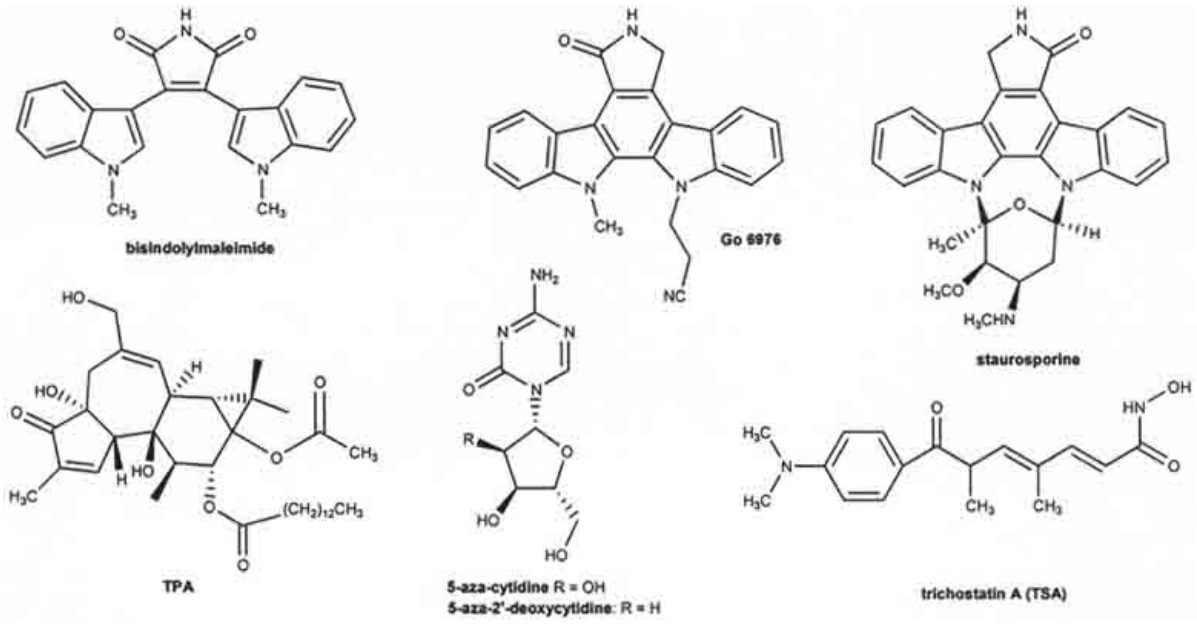

Fig. (5). Regulators of hTERT expression. Chemical structures of natural or chemically synthesized compounds active in the regulation of hTERT expression.

enzymatic activity of telomerase is hTERT [6]. This catalytic subunit is expressed in most malignant tumors but not in normal tissues and hTERT expression is closely associated with telomerase activity, whereas the two other factors are constitutively expressed in both tumor and normal tissues [54, 55, 56]. In addition, introduction of the hTERT gene into telomerase negative cells lead to telomerase expression, telomere elongation and extended life span [57, 58]. Hence, hTERT is a rate-limiting determinant of telomerase enzymatic activity. A more thorough understanding of hTERT regulation may provide not only a molecular basis of cancer progression but also a way to manipulate telomerase activity as a potential therapeutic modality. Recent studies on telomerase regulation showed that telomerase activation is achieved at various steps, including transcriptional and post-transcriptional levels of the telomerase reverse transcriptase (hTERT) gene. A number of transcription factors, tumor suppressors, cell cycle inhibitors, cell fate determining molecules, hormone receptors and viral proteins have been implicated in the control of hTERT expression, as described below [59] (chemical structures are grouped in figure **5**).

PKC Inhibitors

Protein kinase C (PKC) is involved in the regulation of telomerase activity *in vivo* and PKC-modulating drugs alter telomerase activity as reported by a number of papers. Two protein kinase C (PKC) inhibitors (bisindolylmaleimide I and H-7) were found to produce a significant inhibition of telomerase activity in treated nasopharyngeal carcinoma cells NPC-076. On the other hand, staurosporine produced a moderate inhibition, and sphingosine had a small inhibitory effect. The inhibition of telomerase activity by PKC inhibitors appeared to be specific since the treated cells were (> 75%) mostly viable and still retained significant levels of protein synthesis capability [60]. The decrease in telomerase activity by PKC inhibition was not mediated by transcriptional down-regulation of hTERT, as it was shown that PKC participates in the regulation of telomerase activity by direct or indirect phosphorylation of telomerase proteins [61]. Exposure of cervical cancer cell lines (HeLa and CUMC-6) to PKC inhibitors, bisindolylmaleimide I and Go6976, and to high levels of PKC activator, 12-O-tetradecanoyl phorbol 13-acetate (TPA), resulted in the inhibition of telomerase activities by bisindolyl-maleimide I and Go6976. TPA increased telomerase activity at low doses and decreased activity at high doses. The expression levels of human telomerase RNA (hTR) were not influenced by PKC modulating drugs. In contrast, the expression of hTERT decreased after exposure to bisindolylmaleimide I and Go6976 in a time-dependent manner. hTERT expression was not affected by low doses of TPA, whereas it was inhibited at high doses of this drug. [62].

Histone Deacetylase (HDAC) Inhibitors

Emerging evidence suggests that reversible acetylation of nucleosomal histones and the resultant changes in the chromatin structure are important processes in gene transcription. In particular, histone deacetylase (HDAC) inhibitors activate the transcription of certain genes by altering the acetylation status of nucleosomal histones; they are known to modulate transcription and exhibit antiproliferative effects on cancer cells.

Numerous groups sought to investigate whether histone acetylation/deacetylation processes can influence hTERT transcription. Until now the results have been inconsistent.

Two studies showed significant reduction of telomerase activity in human liver cancer cell lines and prostate cancer cell after treatment with sodium butyrate and trichostatin A

(TSA), two potent HDAC inhibitors. In one case, the inhibitory activity did not affect transcription levels of the reverse transcriptase component [63], in the other case HDAC inhibitors down-regulated telomerase activity via suppression of hTERT mRNA expression without affecting cell cycle arrest, apoptosis, or cell differentiation [64].

Other groups, on the contrary, showed that treatment with the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) induced significant activation of hTERT mRNA expression and telomerase activity. Inhibition of histone deacetylases by TSA in telomerase-negative cells resulted in activation of telomerase activity and up-regulation of hTERT mRNA [65]. Similarly, TSA inhibited deacetylation of histones at the hTERT promoter and attenuated the repression of hTERT transcription during HL60 cell differentiation. Also, TSA treatment activated hTERT transcription in resting human lymphocytes and fibroblasts [66]. Both Takamura [67] and Hou [68] demonstrated that TSA induced hyperacetylation of histones at the hTERT proximal promoter, directly transactivated the hTERT gene in normal human telomerase-negative cells, and unregulated hTERT expression in telomerase-positive tumor cells, suggesting that histone deacetylation may be involved in silencing the hTERT gene in normal cells. The responsible element was determined to be the proximal 181 bp core promoter of hTERT, which contains two c-Myc and five Sp1 sites; Sp1, and not c-Myc, resulted to mediate HDAC inhibitor activation of the hTERT promoter.

Demethylatig Agents

Guanine methylation/demethylation processes were also considered as modulating mechanisms of hTERT expression regulation. Indeed the promoter of the hTERT gene encoding the catalytic subunit of telomerase has a dense CG-rich CpG island, suggesting a role for methylation in regulation of hTERT expression. Treatment of SUSM-1 cells with the demethylating agent 5-aza-2'-deoxycytidine induced the cells to express hTERT, suggesting a potential role for DNA methylation in negative regulation of hTERT [69].

On the other hand, 5-aza-cytidine was shown to inhibit telomerase activity via transcriptional repression of hTERT. In fact 5-aza-cytidine treatment of two prostate cancer cell lines, DU-145 and TSU-PR1, significantly reduced telomerase and induced growth inhibition. Inhibition of telomerase activity was accompanied by down-regulation of telomerase catalytic subunit (hTERT) mRNA expression. 5-Aza-cytidine repressed the transcriptional activity of the hTERT promoter, and the core promoter was responsible for this down-regulation [70].

Exogenous Factors

As an exogenous factor, arsenic was found to inhibit transcription of the hTERT gene, by decreasing c-Myc and Sp1 transcription factor activities. Decreased telomerase activity led to chromosomal end lesions, which promote either genomic instability and carcinogenesis or cancer cell death, thus explaining the seemingly paradoxical carcinogenic and antitumor effects of arsenic [71].

Cell-synchronizing agents such as methotrexate, hydroxyurea, and colchicine, caused proliferating cells to cease dividing and become quiescent, but in this case telomerase activity remained essentially unaltered compared to the control cultures [64].

Endogenous Regulators

Little is known on how normal human cells repress telomerase (hTERT) gene expression. However, as the regulatory factors that control telomerase expression are progressively discovered, many more endogenous molecules are found to be involved in the subtle mechanism of up- and down-regulation of telomerase activity.

The studies on the mechanisms of hormonal control of telomerase activity demonstrated that telomerase function in estrogen receptor (ER)-positive MCF-7 cells was upregulated by the treatment with 17beta-estradiol. This activation accompanied up-regulation of the telomerase catalytic subunit, hTERT mRNA and estrogens were shown to activate telomerase via direct and indirect effects on the hTERT promoter [72]. Progesterone, that usually antagonizes estrogen action in reproductive organs, was shown to exert diverse effects on hTERT mRNA expression in a time-dependent manner in breast and endometrial cancer cell lines expressing progesterone receptor. Progesterone significantly induced hTERT mRNA expression within 3 h after exposure. This transient effect peaked at 12 h and then decreased. In contrast, exposure to progesterone for > 48 h antagonized estrogen effects and inhibited the estrogen-induced activation of hTERT expression. Hence, hTERT gene was revealed to be a target of both estrogen and progesterone hormones [73].

The sphingolipid C(6)-ceramide (20 μM) was already known to be involved in mediating important cellular activities such as induction of cell differentiation, growth arrest, senescence and apoptosis, in some human cancer cells [74]. A recent study showed a C(6)-ceramide-mediated significant reduction of telomerase activity correlated with decreased levels of telomerase reverse transcriptase (hTERT) protein and hTERT mRNA in a time-dependent manner. C-Myc protein, a transcriptional factor for hTERT, but not its mRNA levels were decreased in response to C(6)-ceramide at 24 h and the effects of ceramide on the c-Myc protein were shown to be due to a reduction in half-life via increased ubiquitination. Hence, both exogenous and endogenous ceramides were demonstrated to mediate the modulation of telomerase activity via decreased hTERT promoter activity caused by rapid proteolysis of the ubiquitin-conjugated c-Myc transcription factor [75, 76].

Newly discovered endogenous regulators of the telomerase function include the cell cycle regulator p53, which can repress telomerase activity through down-regulation of hTERT transcription where the interaction of p53 with Sp1 in hTERT promoter region was essential [77]; the transcription factor E2F-1, which was shown to function as a transcriptional repressor of the hTERT gene in human cells [78], and the putative hTERT repressor on chromosome 3, which regulated the expression of hTERT without

showing activity on c-Myc or one of its co-regulators [79]. A 400 bp region upstream of the hTERT core promoter was identified to function as a negative regulatory region and the endogenous myeloid-specific zinc finger protein 2 (MZF-2) was found to be an effector for negative regulation of hTERT [80]; PinX1 was shown to bind the telomerase catalytic subunit hTERT and strongly inhibit its activity. Hence, PinX1 represents a potent telomerase inhibitor and a putative tumor suppressor [81].

Finally, the observation that several human tissues as well as some normal cell strains have been shown to express low levels of hTERT mRNA even though they lack telomerase activity, has prompted the investigation on the nature of hTERT found in these cells. Six splice variants of hTERT, including a "deletion" variant (hTERTalpha) that misses conserved residues from the catalytic core of the protein were detected in normal and developing human tissues. Interestingly, hTERTalpha inhibited endogenous telomerase activity, which results in telomere shortening, chromosome end-to-end fusions, senescence-like state in HT1080 cells, and apoptosis in a jejunal fibroblast cell line. These results suggest a role for hTERT splice variants in the regulation of telomerase activity [82]. Recently, expression of a dominant-negative form of hTERT (DN-hTERT) resulted in inhibition of telomerase activity, decrease in mean telomeric length of BEL-7404 human hepatoma cells and elimination of tumorigenicity *in vivo*. These data suggest that hTERT represents an important target for anti-cancer drug screening and DN-hTERT may be employed as a lead structure for the development of antitumor strategies [83].

IMMUNOTHERAPY

Recently, there has been exciting work in the field of antigen-specific immune responses in tumor cells. Initial disappointing trials indicated that the majority of tumors in animal models were non-immunogenic [84]; subsequently, the lack of immunogenicity was linked not to the lack of tumor antigens, but to the inability of tumors to appropriately activate the immune system [85]. This observation has led to the discovery of multiple tumor antigens both for murine and human tumors in the recent years. However, an effective anticancer immunotherapy would require the antigen to be tumor-specific, whereas normal tissues also share most tumor-associated antigens. Furthermore, the antigen should cause “tumor rejection” indicating that it should not be only selectively associated with cancer but also efficiently targeted to destroy tumor cells, leading to clinically significant tumor regression. Hence, the ideal tumor-rejection antigen would: 1) be expressed in most tumors for broad applicability; 2) be expressed restrictedly to the tumor to avoid autoimmunity; 3) not be expressed in the adult organism to avoid the need to overcome tolerance; 4) have a crucial role for the tumor to prevent antigen loss variants; 5) induce an immune response that leads to tumor regression; 6) be an MHC class I and II epitope for the induction of $CD4^+$ and $CD8^+$ T-cell responses [86].

As mentioned before, telomerase is present in the majority of human cancers and is, therefore, a good candidate as a widely distributed tumor-associated antigen. The recently discovered immunological properties of the telomerase reverse transcriptase hTERT suggest that the enzyme is also an attractive target for novel immunotherapies against cancer, given the vast overexpression of hTERT in human tumors and its lack of expression in normal tissues. Clinical trials have begun to test the credentials of hTERT as a broadly applicable target for immunotherapy of cancer. So far data from both human and murine systems demonstrate that cytotoxic T-lymphocytes (CTL) can recognize peptides derived from hTERT and kill hTERT-positive tumor cells of multiple histologies.

MHC Class I Allele Differentiation

A nine-amino acid peptide (I540) derived from hTERT has been shown to bind to human leukocyte antigen HLA-A2.1. This major histocompatibility complex (MHC) class I allele is expressed by 50% of the population, making it an attractive target. By priming with the peptide pulsed autologous dendritic cells, CTL from CD8+ T cells from the peripheral blood of normal HLA-A2.1 donors were generated. A specific CTL response in a variety of tumor cells expressing hTERT was shown and this response was not present in either telomerase negative cells or in cells negative for the HLA-A2.1 allele [87]. The same authors identified hTERT peptide K973, which generated specific CD8+ CTL from HLA-$A3^+$ cancer patients and healthy individuals. These CTL lysed hTERT+ tumors of multiple histologies in an MHC-restricted fashion, suggesting that the epitope was naturally processed and presented by tumors. Since the HLA-A2.1 and the HLA-A3 allele are expressed by 50% and 15-25% of the entire population respectively, these findings extended the potential applicability of hTERT as a therapeutic target to >60% of all cancer patients [88]. Two hTERT-derived peptides (VYAETKHFL and VYGFVRACL) were capable of generating hTERT peptide-specific and HLA-A24-restricted CTL and the CD8(+) CTL clones specific for these hTERT peptides exhibited cytotoxic properties against leukemia cells in an HLA-A24-restricted manner. The HLA-A24 (HLA-A*2402) allele is the most common allele among Japanese (>60%) and is also present in persons of the European descent (nearly 20%). Thus, the above results confirm the feasibility of immunotherapy approaches using hTERT-derived peptides on a wide portion of the world’s population [89].

In vivo Immunologically Significant Antigenes

Although high frequencies of T lymphocytes specific for certain tumor-associated antigens have been detected in some cancer patients, increasing evidence suggests that these T cells may be functionally defective *in vivo* and fail to induce meaningful clinical responses. One strategy to overcome this limitation would be to target novel antigens that are ignored during the natural antitumor immune response but are, nevertheless, capable of triggering effector T-cell responses against tumors after optimal presentation by antigen-

presenting cells. Ayyoub *et al.* reported that hTERT(540)-specific CD8(+) T cells were able to specifically recognize HLA-A*0201 cells either pulsed with peptide or transiently transfected with a minigene encoding the minimal epitope. In contrast, they failed to recognize hTERT-expressing HLA-A*0201(+) target cells [90]. Another work demonstrated that the (hTERT)-tumor antigen identified by epitope deduction rather than from patient immune responses, was immunologically ignored by patients despite progressive tumor burden. Nevertheless, HLA-A2-restricted CTL against hTERT were equivalently induced *ex vivo* from patients and healthy individuals and efficiently killed human tumor cell lines and primary tumors. Thus, telomerase-specific T cells from cancer patients were spared functional inactivation because of immunological ignorance [91]. These findings support clinical efforts to target the hTERT as a tumor antigen with broad therapeutic potential**.**

Immune System Antigene Presentation

The above results indicate that the way the cancer-derived antigens are presented to the immune system is essential for a correct and significant response against the tumor. Dendritic cells (DC) are rare cells with a unique ability to take up, process and present antigens in a highly efficient manner, as key players in the primary immune response and even in the pathogenesis of autoimmunity. Moreover, it is now clear that DC activation is the common pathway in the initiation of an antitumor immune response elicited by a variety of cancer vaccines. DC can be transfected with DNA or RNA encoding tumor antigens or physically loaded with corresponding proteins or peptides derived from tumor extracts [92] or cell lysates [93]. Pulsed transfection of DC with RNA or DNA is preferred to antigen transfection: RNA can be functionally amplified using PCR technology, and non-limiting amounts of antigen can be generated even from small amounts of tumor tissue, while antigen preparation for direct antigen pulsed transfection of DC would be hampered by the requirement of large amounts of tumor tissue and the need to identify the antigens involved [94]. Once the engineered DC have been infused back into the host, the assumption is that they will traffic to specialized microenvironments of secondary lymphoid organs, where the activation of immune responses originates [95].

To date, it has been shown that hTERT RNA transfected DC can remarkably be effective in stimulating CTL and tumor immunity in both *in vitro* and *in vivo* models. Immunization of mice with hTERT RNA-transfected DC stimulated CTL, which lysed melanoma and thymoma tumor cells and inhibited the growth of three unrelated tumors in mice of distinct genetic backgrounds. Furthermore, hTERT RNA-transfected human DC stimulated hTERT-specific CTL *in vitro* that lysed human tumor cells, including Epstein Barr virus (EBV)-transformed B cells as well as autologous tumor targets from patients with renal and prostate cancer [96, 97].

Recent evidence, however, showed that the tumor-specific CTL were consistently superior to the CTL stimulated with hTERT RNA-transfected DC in recognizing and lysing tumor targets, suggesting that tumor-specific CTL represent a polyclonal response providing more effective antitumor activity than T-cell responses directed against a single antigen in the form of hTERT. Tumor RNA-transfected DC were capable of stimulating T-cell reactivities not only against the primary tumor but also against metastatic tumors, although discrete differences in the antigenic repertoire expressed by these tissues were apparent [98]. Thus, total tumor RNA-transfected DC may be a better alternative to hTERT RNA-transfected DC and represent a broadly applicable vaccine strategy to induce polyclonal and potentially therapeutic T-cell responses in cancer patients.

CONCLUSIONS

Four different “alternative” approaches to anti-telomerase therapy were considered in this review.

The first deals with the development of compounds, originally tested as anti-viral agents, which are able to recognize the hTERT catalytic site. Initially, the evidence of hTERT belonging to the reverse transcriptase family of enzymes fueled the hopes to block its activity via substrate analog inhibition. However, the lack of specificity thus far experienced has reduced the enthusiasm in this field.

Secondly, a number of miscellaneous molecules were also found to efficiently interfere with telomerase activity, most of which are thought to act through inhibition of the hTERT subunit or the hTERT/hTERC interacting domains. In this connection, high-throughput screening on random libraries of small molecules appears to represent a powerful approach to the discovery of high-potency novel compounds.

Thirdly, the biophysical and biochemical properties of the telomerase ribonucleoprotein complex and its regulating mechanisms are progressively being unveiled. This will shed further light on the participation of established drugs in interference with telomerase-dependent cellular pathways. On turn, the new information will surely be of great help in the rational design of telomerase-based specific drugs. It is also likely to open new avenues for the design of unique families of protein inhibitors acting upstream the telomerase cascade.

Finally, the discovery that hTERT-derived peptides are highly immunogenic and induce $CD4^+$ and $CD8^+$ T-cell responses producing tumor cell lysis, opens new perspectives to the production of effective cancer vaccines.

Together with G-quadruplex, antisense and rybozyme-based approaches, the strategies presented here make us confident that telomerase and the factors controlling its expression represent suitable targets for successful developments in cancer chemotherapy. The years to come will certainly witness outstanding progress in this field.

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