

**PHARMACOLOGICAL REGULATION OF
TELOMERASE: INDUCTION OF TELOMERASE
DEPENDENT CELL DEATH USING
CHIMERIC OLIGONUCLEOTIDES
OR ALL-TRANS-RETINOIC-ACID/ARSENIC-TRIOXIDE
COMBINATION TREATMENT**

ILONA TARKANYI

DOCTORAL (Ph.D.) THESIS

University of Debrecen, Medical and Health Science Center,
Department of Biochemistry and Molecular Biology
&
Universite Paris XI,
Faculte de Medecine Paris-Sud

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INTRODUCTION

It was more than 30 years ago when first questions were raised about the special problem concerning the replication of the linear chromosomes' end. Incomplete replication of the 5'-end, thus progressive loss of chromosomal sequence seemed to be the theoretical barrier of unlimited proliferation. The answer followed in 1984, when an enzyme responsible for the replication of the lagging strand's end was discovered. For a period of time we thought to have the answer to all our questions: apparently we had the key to unlock all the secrets of immortality, a basic feature of unlimited growth, thus neoplastic diseases. The new enzyme, termed telomerase, has been announced as a universal cancer target, and the solution for treatment of malignant proliferative diseases seemed to be just as simple as finding the appropriate inhibitor. The following decades passed with changing enthusiasm and disappointment. Fact is, that even today, our knowledge about regulation, molecular interactions and functions of telomerase is very limited and as a consequence, inhibitors and inhibition strategies did not progress to clinical application. Research of the pharmacological regulation of telomerase has become a sober minded field, with more moderate expectations. The challenge, however, is big: basic research, as well as pharmaceutical industry are seeking for a way to have the control over life span of living cells.

TELOMERES

It has been clear since the early decades of modern cell biology that living cells have to be able to distinguish between the physiological end of a linear chromosome and a double strand break created by an injury. Special genetic structures at the end of the chromosomes, termed “telomere” from the Greek words “telos”, meaning “end” and “meros” meaning “part”, were described long before their significance and function was discovered.

Telomeres are terminal protein-DNA complexes that stabilize chromosomal ends and prevent them from aberrant recombination and being recognized as DNA double-strand breaks (1). Besides the classical function to protect the chromosome end, they have an important role in chromatin organization and cell proliferation control. They consist of repetitive DNA sequences, in mammals 5'-TTAGGG-3', and specific protein complexes. Telomere length is species specific: human telomeres usually extend to 5-15 kb length. Modest in-species variability might exist, as well as variation due to the nature of the tissues, the age and the normal or pathological status of the cells. The 3'G-rich extends beyond the double helix and forms a single-stranded overhang with about 150-200 nucleotides (2).

Human telomeric DNA loops back on itself forming a t-loop (telomere-loop), with the G-rich tail invading the duplex and forming a D-(displacement) loop. This structure, stabilized by telomeric proteins, seems to exist to protect against nuclease degradation and recombination. Moreover, telomeres in this folded structure seem to be prevented from elongation by telomerase. A recent hypothesis suggests that before telomerase was developed by nature, t-loop was alone responsible for telomere maintenance (3).

Another possibility to eliminate G-tail function of the telomeres and regulate telomerase is the alteration of the overhang structure. DNA sequences that contain stretches of guanines can form four-stranded structures called G-quadruplexes. This type of telomere folding was demonstrated first *in vitro*, but now also found *in vivo* in *Stylonychia lemnae macronuclei* (4). There is yet no demonstration about their existence in human, and the physiological role has also not been proven.

TELOMERE MAINTAINING MECHANISMS

DNA-polymerases replicate DNA only in the 5' to 3' direction and cannot initiate synthesis of the DNA chain *de novo*, just with a small RNA stretch as primer. Normal lagging-strand DNA replication fails to copy the 5'-end of the chromosome, thus leaving a gap between the final RNA priming event and the terminus. This leads, in the absence of a compensatory mechanism, to progressive telomere shortening at each cell division with 50-200 basepairs. This phenomenon has been termed as “end-replication problem”. It is clear that progressive loss of the genetic material is incompatible with life, thus organisms must have developed methods to reconstitute their telomeres. The repetitive structure already suggests the possible mechanisms: a processive DNA-polymerase that adds *de novo* repeats or homologous recombination.

Telomerase

Telomerase is a ribonucleoprotein complex that includes an RNA template (hTR) and a catalytic subunit (hTERT) with reverse transcriptase activity. Telomerase binds the 3' overhang of the telomeric DNA, and extends it by copying its RNA-

template that is complementary to the hexameric unit of the DNA telomeric repeat sequence (5). Telomerase has been shown to add repeats to the telomeres in a processive manner (6): the 3' end of the telomere binds to the template region of hTR and is elongated by the addition of bases complementary to the template via the catalytic subunit, the complex then translocates and repeats the elongation step.

Alternative Lengthening of Telomeres (ALT)

Telomere maintenance was long time associated exclusively with telomerase. In 1994 the existence of a telomerase-independent maintenance of mammalian telomeres was described, and termed "Alternative Lengthening of Telomeres" (ALT). Telomeres in ALT cells are maintained via homologous recombination: DNA strand from one telomere anneals with the complementary strand of another telomere, thereby priming the synthesis of new telomeric DNA using the complementary strand as template (7). ALT-positive cells are characterized by telomeres very heterogeneous in length (ranging from very short to long) as well as the so-called associated PML (Promyelocytic leukemia) bodies (APB). APBs are nuclear structures containing telomere DNA, telomere binding proteins (TRF1 and TRF2), a variety of proteins involved in DNA repair, recombination, replication and PML (8). New findings show that telomere recombination exists as well without the above characteristics (9)

Majority of epithelial tumors as well as normal cells with replicative capacity rely on telomerase, ALT is more often present in cell lines and tumors of mesenchymal origin. Coexistence of ALT and telomerase seems to be possible but was not yet clearly demonstrated in vivo. Recent findings suggest the possibility that human tumors may be able to reversibly convert their telomere maintenance phenotype from a telomerase-dependent to a telomerase-independent mechanism (10). A predicted consequence of the

existence of ALT in some human tumors is that telomerase inhibitors will be ineffective in this tumor subset, and that use of telomerase inhibitors for cancer treatment will provide a selective advantage to cells which activate an ALT mechanism.

REGULATION OF TELOMERE LENGTH AND STRUCTURE:

TELOMERIC PROTEINS

Organization of telomeric DNA is different from the rest of the chromosome, thus DNA-associated proteins are as well special ones. Telomeric proteins play important roles in regulating telomere length, integrity and function. Main factor in telomere length regulation is Telomeric Repeat binding Factor 1 (TRF1), which binds as dimer to the double-stranded TTAGGG sequence. The crude model of its action is the following: long telomeres recruit a large number of TRF1 proteins, which inhibits telomerase in adding more repeats, whereas short telomeres with less TRF1 have a higher chance to be elongated. Overexpression of wild-type TRF1 or the expression of the dominant-negative mutant form of TRF1 was shown to induce telomere shortening or elongation , respectively, until the setting of a new equilibrium (11).

Binding of TRF1 to the telomeres can be inhibited by Tankyrase 1 (TRF1-interacting ankyrin-related poly-(ADP-ribose)-polymerase 1) and Tankyrase 2. ADP-ribosylation of TRF1 by tankyrases enables telomerase to access the telomeres and elongate them (12). TIN2 (TRF1-interacting nuclear protein 2) stabilises TRF1-tankyrase interaction, and acts as negative regulator of telomere length by causing conformational changes in TRF1 protecting it from being modified by tankyrase (13).

hPOT1 (Protection of telomere 1), a single-stranded DNA binding protein with great sequence specificity for telomeric repeats, plays also a critical role in maintaining telomere integrity. This protein transduces the information about telomere length to the terminus where telomerase exerts its activity. TRF1 complex recruits POT1 to the telomeric chromatin, and POT1 inhibits telomerase directly or indirectly through the sequestration of the 3' terminus (14). New findings, however, suggest a more complex role of hPOT1 in telomere maintenance: to disrupt G-quadruplex structures in telomeric DNA, thereby allowing proper elongation by telomerase (15).

Telomeric Repeat binding Factor 2 (TRF2) is found in the double-stranded part of the T-loop and in the loop-tail junction, and stabilizes the specific two-loop structure formed by the sequestered 3' overhang. Inhibition of TRF2 results in loss of the single stranded termini and induction of the DNA damage pathway finally leading to apoptosis (16). TRF2 associates with several proteins involved in DNA damage and repair responses, notably RAD50/MER11/NBS1 complex, a key component of the homologous recombination and non-homologous end-joining pathways, the ERCC1/XPF nucleotide excision repair endonuclease, as well as ATM kinase, the WRN (Werner protein) and BLM (Bloom protein) helicases (reviewed in (17)). Recent results demonstrated that POT1 and TRF2 share in part the same pathway for telomere capping and suggested that POT1 binds to the telomeric single-stranded DNA in the D-loop and cooperates with TRF2 in t-loop maintenance (18).

STRUCTURE, REGULATION AND FUNCTION OF THE TELOMERASE HOLOENZYME

Essential components of the telomerase holoenzyme are the human telomerase RNA (hTR) with a well defined secondary structure, and the catalytic subunit, the human telomerase reverse transcriptase (hTERT).

STRUCTURE OF THE TELOMERASE COMPLEX

hTERT (human Telomerase Reverse Transcriptase)

The catalytic component of the telomerase enzyme, located at the end of the chromosome 5 (5p15.33), is a DNA polymerase with reverse transcriptase activity. The amino-terminal moiety of the 127 kDa protein is essential for the nucleolar localization, and multimerisation. The COOH-terminal region is involved in the processivity of the enzyme and is indispensable for in vivo activity (19). The central region contains the motifs characteristic of reverse transcriptase proteins (1, 2, A, B', C, D, and E) and a conserved RNA-binding domain, required for specific binding of hTR by the hTERT subunit (20). Domains attributed to the classical functions of telomerase are relatively well established, but protein parts corresponding to novel functions, like anti-apoptotic activity or nuclease activity (21) serving a probable proofreading role, are almost unknown.

hTR (human Telomerase RNA)

The human telomerase RNA (hTR) extends on 451 nucleotides and contains the 11 nucleotide long template sequence for telomeric repeat synthesis. The half-life of this RNA, transcribed from 3q21-q28 locus by RNA polymerase II, varies from 4 to 32 days

(22), and is the longest ever reported for a eukaryotic RNA. Processing and stability of hTR require binding of nucleolar RNA binding proteins, for e.g. dyskerin. Although sequence homology between different organisms is limited, secondary structure seems to be conserved within families (23). Conserved regions in vertebrates include H/ACA type domain (CR6-8) at its 3' end and other domains, like the template, pseudoknot (CR2-3), CR4-5, CR7, which are involved in hTR stabilization, its accumulation, its assembly with other components and its cellular localization (Fig.1).

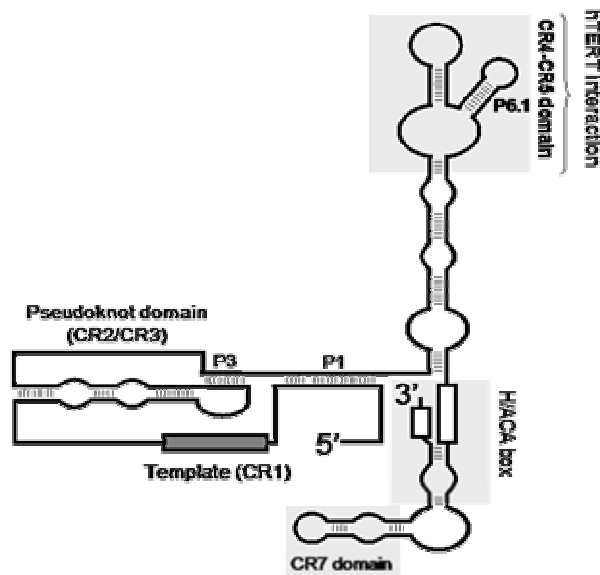


Figure 1. :Structure model of telomerase RNA. The vertebrate telomerase RNA consists of four universally conserved structural elements: the pseudoknot domain, CR4-CR5 (conserved region 4 and conserved region 5), box H/ACA and CR7 (conserved region 7) domain.

The interaction of hTR with hTERT determines the catalytic activity, processivity, telomere binding and has a role in proper ribonucleoprotein folding and assembly as well. Like other snoRNP complexes, telomerase assembly occurs in the nucleolus.

REGULATION OF TELOMERASE

Telomerase is active during embryonic development, then gradually repressed during differentiation in the majority of somatic cells. In adult, it is detected only in certain stem cells of renewable tissues. Telomerase repression is thought to act like a

tumor suppressor mechanism. In normal fibroblasts low telomerase activity is present in S phase, most probably to maintain telomere structure, thereby preventing premature ageing due to telomere dysfunction (24).

Since deficiency as well as overexpression are pathogenic, telomerase has to be fine regulated. Although both essential telomere components are targeted by several regulatory mechanisms, hTERT seems to be the rate-limiting factor in the regulation of telomerase activity.

Transcriptional regulation of hTERT

Continuous telomere maintenance is predominantly due to the transcriptional upregulation of hTERT expression, which is governed by complicated regulatory pathways. Most important regulatory mechanisms influencing hTERT transcription include nuclear hormones, transcription factor families, like E-box binding proteins and ETS proteins, viral enhancers and several tumor suppressor proteins (Fig. 2).

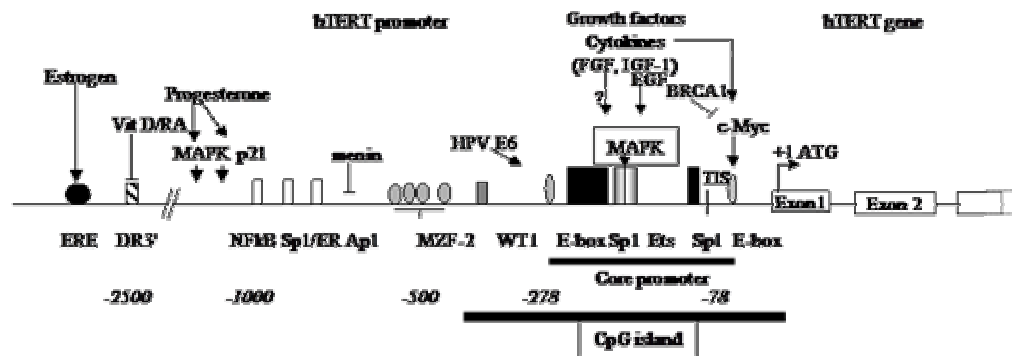


Figure 2.: 5' regulatory region of hTERT summarizing some of the binding sites of transcription factors. TIS = transcription initiation site, ATG = translation initiation codon.

Estrogen activates telomerase in several cell types, first by interacting with the estrogen response element in the distal promoter, second by a stimulatory sequence near the recognition site of Sp1 transcription factor (25). According to this, Tamoxifen, a known anti-estrogen has been shown to downregulate telomerase in some cells, for e.g. in cancer derived from mammary epithelium (26). Progesterone has also been shown to activate telomerase, although molecular backgrounds are still unclear (27) similar to androgens where the delayed response is likely due to an indirect effect (28). Repressory activity has been proven for the combination of vitamin D₃ and 9-cis-RA, which inhibited telomerase activity through direct interaction of the heterodimer of vitamin D receptor (VDR) and RXR with the specific response element in the hTERT promoter (29).

Transactivation and repression by Myc/Max/Mad network of transcription factors is mediated by binding to the two E-boxes within the hTERT promoter and seems to have a crucial role in the regulation of telomerase. Max can form heterodimers with Myc and Mad, resulting in gene activation (Myc/Max) or repression (Mad/Max). Reciprocal occupation of the E-boxes by the different heterodimers is due to inversely regulated expression levels of the family members. c-Myc is known to be activated in proliferating and many neoplastic cells. Switching from Myc/Max to Mad/Max at the promoter is accompanied by hTERT downregulation (30), whereas the reverse switch has been noted with acquisition of telomerase activity. The amount of c-Myc can be regulated at gene level by several cytokines for e.g. the transforming growth factor- β (TGF- β) or by factors binding the protein, like the tumor suppressor BRCA1 (31). Core promoter of hTERT contains besides the E-box, several canonical and degenerate binding sites for the transcription factor Sp1 (32). The role of Sp1 in endogenous

hTERT regulation is not clear since its function to activate or repress seems to depend on the promoter context (33).

ETS binding sites (EBS) are highly conserved between the human and mouse hTERT promoters. Despite of this, the significance of Ets transcription factors is not clear yet. One of the strongest evidences for their role in telomerase expression was found in Ewing's sarcoma, where the fusion oncoprotein EWS/ETS was shown to activate the transcription of hTERT (34).

Activation of hTERT has also been observed in oncogenic viral infections. As a good example, the HPV E6 protein can also associate with c-Myc and thereby activate hTERT transcription (35).

In addition to these inducers of telomerase activity, negative regulators of hTERT transcription have also been described, including factors already mentioned like, Mad1 and the TGF- β regulated transcription factor SIP1 (Smad-interacting protein), as well as known tumor suppressors as the Wilms' tumor 1 protein (WT1) myeloid specific zinc finger protein 2 (MZF-2) (36) and Menin (product of MEN1 tumor suppressor, mutated in multiple endocrine neoplasia). More general repressor candidates are p53, pRB and E2F (reviewed in (37)).

Obviously, regulation of hTERT transcription is quite complex, and in many details still obscure. Making it even more complicated we should not forget that the chromatin context as well plays an important role in the regulation of telomerase.

Epigenetic regulation of hTERT promoter

Epigenetic mechanisms, like DNA-methylation and histone postranslational modification, thus changes in the chromatin structure represent an important level of gene expression, and emerging evidence suggests their implication in hTERT

regulation. Histone modifications are likely to control the structure and/or function of the chromatin fiber, with different modifications yielding distinct functional consequences. Site-specific combinations of histone modifications were shown to correlate well with particular biological functions. In case of the telomerase catalytic subunit the importance of chromatin remodeling has been shown on several models.

In normal cycling fibroblasts, transitoral induction of hTERT was linked to assembly and disassembly of E2F-pocket protein-histone deacetylase complex (38).

During differentiation, as shown in HL-60 cells, the switch of c-Myc to Mad1 can induce active deacetylation at the hTERT promoter, leading to rapid decrease in hTERT mRNA. (30)

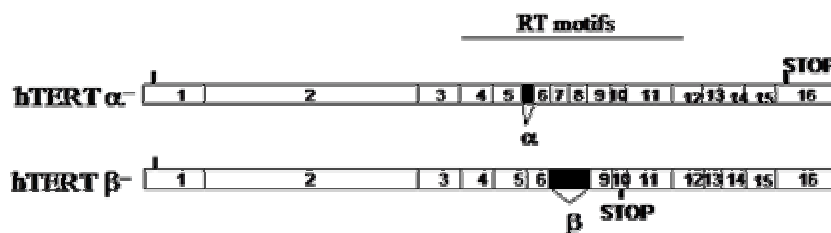
Histone modifications have been implicated in telomerase repression in ALT cells: lack of expression of hTR and hTERT seems to be associated with histone H3 and H4 hypoacetylation and methylation of Lys9 on histone H3 (39).

The presence of CpG islands on hTERT promoter suggested that methylation can play an important role in the regulation of hTERT transcription in normal and cancer cells. However, experiments to discover the relationship between hTERT promoter methylation and telomerase expression lead to contradictory results. Surprising first findings demonstrating a positive correlation between hypermethylation of the hTERT promoter and hTERT mRNA expression (40), initiated the systematic evaluation of the methyl-cytosines in the CpG island surrounding the hTERT promoter. Promoter region from -500 to +1 (including core promoter region, see Fig. 2) revealed a clonal hypermethylated pattern in tumor tissues and cell lines, and a totally unmethylated pattern in normal tissues. In this region of the hTERT promoter, a correlation between methylation and gene expression was defined (41). A decrease of hypermethylation was observed in the first hundred bases of the proximal exonic region

in telomerase-positive cells, followed by a complete methylation. Indeed, reporter constructs showed already before, that exon1, plays an important role in the regulation of hTERT. What seems to be clear now, is that hTERT might teach us new lessons in epigenetics.

Alternate splicing

Alternate splicing of hTERT transcripts seems to be another regulatory mechanism for telomerase. Growing knowledge of telomerase biology identified several processes where the amount of catalitically active protein was regulated by splicing. Hypoxic conditions for example, involve hTERT regulation by alternative splicing, which involves a switch in the splice pattern in favor of the active variant (42). TGF- β 1 was able to modulate the splicing pattern of a skin keratinocyte line, by shifting its expression from the full-length active form to the inactive smaller β -variant (43). Regulation through splicing is not restricted to pathological conditions, like tumors: splicing during human development was shown to occur in tissue and gestational age specific patterns (44). Systematic evaluation of telomerase positive cell lines showed that potentially functional form accounts only for 5% of the total message (45).



. Figure 3.: hTERT splice variants: α and β deletion

Besides the potential active form, several deletion-type splice variants and a number of insertion-type molecules have been reported for hTERT. The variants with α and β deletions are the best characterized. Both of them remove regions of the conserved reverse transcriptase domain of hTERT, thus none of them encode a catalitically active protein (Fig. 3)

hTERT postranslational modifications

As in case of many enzymes, postranslational modifications are used in some cell types to control hTERT activity. hTERT phosphorylation by serin/threonin or tyrosin-kinases has been shown to play a role in several pathological and physiological processes.

During megakaryocytic differentiation, which is uniquely accompanied by DNA replication, a transient increase in TA can be observed in cell culture models. This is caused by hTERT-phosphorylation, can be inhibited by PKC inhibitors, and is suggested to have an importance in lineage specific differentiation (46).

In T lymphocytes, that express hTERT constitutively, activation causes phosphorylation and nuclear translocation. This process seems to be important for the telomerase activation that accompanies clonal expansion (47).

Besides protein kinase C and B dependent activation, c-Abl dependent inhibition seems to play a role in telomerase biology. The latter could be responsible for the low telomerase activity found in chronic myeloid leukemia harboring a constitutively active kinase due to Bcr-Abl fusion (48) .

Regulation of hTR

Although hTR does not seem to be the rate limiting factor for telomerase, the difference found in its levels between cell types indicates that acquisition of telomerase activity is associated with an active regulation of the RNA component as well, which results in an higher steady-state level of hTR in telomerase-positive cells. Modest information gained about hTR regulation shows that its level is determined by transcriptional and posttranscriptional mechanisms. Immortalization of cells, causing telomerase derepression, resulted in upregulation of hTR transcription, which could not be reproduced by the introduction of exogenous hTERT, suggesting the existence of a regulatory mechanism that coordinates the expression of the telomerase holoenzyme subunits (22). Another mechanism, that proved to account for the higher levels of hTR in telomerase positive cells is the prolonged half-life in the presence of the protein subunit (22). Our logic suggests that there must be a molecular machinery that synchronizes the expression of the protein and RNA component, like for other enzymes with prosthetic groups, but coordinating factors are not elucidated yet.

Regulation of telomerase by chaperones

The assembly of the holoenzyme and the formation of the active telomerase is controlled by several chaperone proteins (49). While the interaction with Hsp70 protein is transitory, the Hsp90 and p23 proteins remain permanently associated to the telomerase complex affecting its activity.

ROLE OF TELOMERASE

Classical role of telomerase is to maintain telomeres, by copying the template sequence of its RNA moiety. This process is regulated by the fine interaction of the telomeric proteins and telomerase itself. Primitive hematopoietic cells show progressive telomere shortening with a low telomerase expression (50), thus it has been suggested that the low telomerase activity is insufficient to maintain telomeres. Experimental overexpression of hTERT showed in contrast, that elevated telomerase activity alone can not stabilize the telomere length (51). This indicates that telomere biology is more than regulation of the telomerase- but it is also clear now that telomerase knows more than just synthesizing telomeric sequences.

RT domains are essential for the telomere maintaining function, but the large hTERT protein contains conserved domains as well outside this regions. Furthermore immortal cells prefer telomerase over alternative mechanisms. This indirect evidences suggested additional functions (often referred as “extracurricular activities”) long before the first observations that telomerase could contribute to install an immortal phenotype by preventing apoptosis. This new function of telomerase can be mediated by its action on the regulation of anti-apoptotic and growth controlling genes (52, 53).

TELOMERASE IN CLINICALS

TELOMERASE AND SENESCENCE

Proliferation of telomerase negative cells results in progressive telomere shortening. When telomeres reach a critical length, proliferation will be irreversibly arrested in G1 phase of the cell cycle, and cells acquire a characteristic enlarged morphology accompanied by biochemical changes, including the activation of the β -

galactosidase active at acidic pH. This process, termed replicative senescence, is believed to be signaled by a DNA damage repair signaling program. However, telomere lengths in most senescent cells have been reported to be 5–10 kb long, thus telomere length alone seems not to induce the senescent phenotype. On the other hand, recent observations in senescent fibroblasts include speculations that erosion of the single-strand overhang plays a role in replicative senescence (54). In vitro cultured cells escape the initial growth arrest and divide until they enter crisis, when telomeres become extremely short, and apoptosis occurs. Furthermore, it has been shown that the shortest telomere is critical for cell viability (55).

Cellular senescence is thought to serve as a protecting mechanism against cancer promoted by immortalization, but consequent telomere dysfunction will be involved in tumorigenesis late in life.

TELOMERASE AND CANCER

Telomere hypothesis of cancer

Telomerase is activated in more than 85% of malignant tumors (56). In contrast, telomerase activity is usually not detectable in normal somatic tissues. Loss of telomere capping, thus telomere dysfunction results in genomic instability observed in early malignant lesions. Loss of genomic integrity leads to dysregulation of genes involved in growth control. Genetic alterations must occur in multiple pathways to convert normal cells to cancer: telomere maintenance can be one of them, since it provides a clear advantage in survival.

Telomerase as tumor marker

Using the difference in telomerase expression between tumor and normal cells, the detection of telomerase activity or telomerase components may be useful as potential novel diagnostic marker for a wide range of cancers (57). Experience of the past years shows that telomerase, despite of being a quite common feature of malignant proliferative diseases from different histological origins, will not be able to replace other tumor markers, thus the telomerase-based diagnostic tests should be used in association with other well-established methods. Telomerase measurements also have potential as a prognostic tool in patients with cancer. Detection of either telomerase activity or protein components in tumor tissues usually means a less favorable prognosis. This could be explained by the fact that telomerase confers a proliferative advantage to malignant cells, and was shown to be responsible for chemo resistance, because of its antiapoptotic role. Positive correlation of telomerase with poor prognosis and/or therapy refractoriness has been demonstrated for solid tumors like breast cancers (58), or hematological malignancies for e.g. AML (59). Measurement of telomerase activity in easily obtained fluids might be as well a useful tool for diagnosis and monitoring (60). As a good example, TRAP-assay from urine probes seems to be a good screening test for urinary bladder cancer in high risk populations (61).

Using telomerase as tumor marker raises some technical problems. Attention should be paid to false negative results due to the instability of the enzyme, the hTERT mRNA, the existence of polymerase chain reaction inhibitors (as most of the activity detection methods use PCR), and the specificity of hTERT antibodies (in immunohistochemistry methods). False-positive results can occur in pathologic specimens with significant lymphocytic infiltrations (62).

TELOMERES AND NON-MALIGNANT DISEASES

Dyskeratosis congenita was the first primary telomere maintenance disorder identified in human. Its characterized by the mucocutaneous triad of skin hyperpigmentation, oral leukoplakia and nail dystrophy as well as progressive aplastic anemia. Autosomal dominant cases result from mutations in the H/ACA domain of hTR, while the X-linked forms result from mutations in dyskerin which impair telomerase assembly. Both of these mutations lead to defective telomere maintenance in stem cells with proliferative activity. Recently a null mutation in motif D of the reverse transcriptase domain of the protein component of telomerase was associated with the autosomal dominant form, which underlines the significance of telomerase function in this disease (63).

Mammalian telomeres are also bound by nucleosome arrays, containing histones that have undergone specific modifications that are characteristic of constitutive heterochromatin. Telomeric heterochromatin can influence the transcriptional silencing of the neighboring genes: this phenomenon is known as telomere position effect (TPE). TPE has been made responsible for some human diseases like *facioscapulohumeral muscular dystrophy* (64).

Mutations in TRF2-interacting telomeric proteins, leading to chromosomal instability, are usually characterized by *premature ageing syndromes*. It is likely that the presence or critically short telomeres is an important part of the disease presentation in cases of Ataxia telangiectasia (ATM), Werner syndrome (WRN); Bloom syndrome (BLM), Nijmegen breakage syndrome (NBS) and ataxia telangiectasia-like disorder (MRE11) (17).

TELOMERASE BASED THERAPY

The relative specificity and high frequency of telomerase expression in wide variety of cancers highlight the potential of telomerase and telomeric structure as targets of anti-cancer therapies. Strategies under development include drugs that directly target either telomerase and telomeres or the telomerase-associated regulatory mechanisms, approaches targeting telomerase-positive cells as telomerase immunotherapy and telomerase promoter driven gene therapy.

TUMOR SPECIFIC IMMUNOTHERAPY

By immunizing patients against hTERT T-lymphocyte mediated killing of tumor cells can be provoked. hTERT-specific immune response has been generated by priming dendritic cells with telomerase RNA, and strong cellular immune response could be obtained without serious side effects (65).

TELOMERASE-MEDIATED TUMOR SPECIFIC GENE THERAPY

Genes placed under the control of hTERT promoter suggested to be expressed in malignant cells but not in normal cells. The hTERT proximal promoter was used to produce tumor specific expression of therapeutic genes, such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), caspase-6/8 or Fas-associated protein with death domain (FADD) (reviewed in (66)). Using these constructs, the growth of tumors in mouse models was significantly suppressed. This strategy has also been used to express enzymes capable of activating prodrugs into cytotoxic drugs in malignant cells. Administration of the prodrug leads to selective killing of cancer cells. Interestingly, suicide constructs using hTR promoter were found in some cases stronger in efficacy and equal in specificity compared with those using hTERT (67).

TELOMERASE INHIBITORS

hTR targeting

hTR was a quite obvious target for telomerase inhibition strategies, as its function as template makes it accessible also for inhibitory strategies. Further insights into telomerase biology, exploring the tumor promoting action of the catalytic subunit independent from telomeric repeat synthesis, raised first doubts for the rationality of such approaches. Recent findings, however, bring anti-hTR treatments back to the frontline, as the RNA component seems to be indispensable to mediate the tumor promoting effect of hTERT overexpression (68).

Although the interest in oligonucleotide-based anti-cancer treatments shows a decreasing trend, the number of the members of the anti-telomerase/hTR oligomers, referred as “antisenses” or “antagonists”, is continuously growing (Table 1). Most oligonucleotide type inhibitor-constructions are complementary to the hTR template site, but research of the past years proved the biological activity of particular non-template directed sequences as well. The advantage compared with conventional antisense approaches is, that in contrast to mRNA-s, hTR-interacting molecules can function as enzyme-inhibitors, by impairing hTR template function and disrupting telomerase holoenzyme integrity, and these effects are independent from RNase H-mediated degradation or the translational machinery. The unfavorable chemical properties of classical phosphodiester DNA-oligonucleotides were a challenge to develop chemically altered oligomers. Chemical modifications of telomerase inhibiting oligonucleotides meant usually altered backbones and were mainly addressed to improve inhibitory parameters, stability, internalization and subcellular delivery. Trials were made with partially and fully phosphorothioate-DNA (DNA_{PS}) (69), peptide

nucleic acid (PNA) (70,71), 2'-O-alkyl (methyl or 2-methoxyethyl) RNA (2-Ome/2'-OHOE-RNA) (70,72), N3'→P5' phosphoramidate (NP) or thio-phosphoramidate (NPS) (73,74) oligomers. Other efforts to enhance stability of antisenses are focused on oligonucleotide structure. Ribbon antisenses (RiAS) consist of two identical antisense loops at both ends and a stem interconnecting the loops. The covalently closed structure resulted in an enhanced stability of hTR-RiAs, and was shown to cause degradation of hTR and subsequent cell death (75).

It has been shown that some of the above types of oligomers are able to cause decrease in telomerase activity, progressive telomere shortening and cell death only in particular conditions, while they fail to be biologically active in others. One of the problems, still without a breakthrough solution, is the cellular uptake of the compounds, raising attention to the fact that IC₅₀s from cell-free experiments might not be informative for cellular efficacy. Several augmenting methods were tested for oligonucleotides in terms of successful telomerase inhibition: various liposomal transfection agents(70,76,77,78), DNA-Tat peptide-lipid complex (DPL-complex) (75) electroporation (71), and for PNA molecules, that represent a special problem having uncharged backbones, photochemical internalization (79), and Antennapedia peptide conjugation (80) as well. hTR template antagonist siRNA (76,81) needed a lentiviral delivering/expressing system to be effective. Despite of all these difficulties, new molecules are developed, for e.g. containing a palmitoyl lipid, conjugated covalently to an aminoglycerol thiophosphate linker (both developed by Geron, Menlo Park, California), that were shown to be potent and well-tolerable anti-cancer agents *in vivo* tumor models. First clinical trials for these compounds are in preparation.

Among the hTR antagonist molecules, not directed against the template region, the most effective ones are those targeting regions of hTR that are responsible for

holoenzyme complex assembly. 2'-O-methyl RNAs (2-OMe-RNA) complementary to the pseudoknot domain at the P3/P1 intersection and the CR4-5 domain at the P6.1 stem-loop proved to be inhibitory on telomerase with IC_{50} s in nanomolar range when added prior to the assemblage (82). A successful construct against non-template hTR was a 2'-5' oligoadenylate-antisense (2-5A-anti-hTR) designed to activate RNase L mediated degradation of the target. This oligomer caused decreased cell viability and apoptosis *in vitro*, and was effective *in vivo* against several subcutaneous tumor models and intracranial gliomas in nude mice as well (83). 2-5A-anti-hTR seems to stimulate the apoptotic pathways regardless of telomere length.

Ribozymes, catalytic RNA molecules capable of site-specific cleavage of target RNAs, designed against the RNA component, were also able to induce telomere shortening and apoptosis in certain *in vitro* cell cultures (84).

Targeting the catalytic subunit

Taking its activity as reverse transcriptase, first inhibitors used to target hTERT were nucleoside analogs that were successfully applied to inhibit viral reverse transcriptases. AZT (3'-azido-2',3'-dideoxythymidine), AZT-TP (AZT-5'-triphosphate), and ddG (2'-3'-dideoxyguanosine) decreased telomerase activity and caused telomere shortening, but it was not sufficient to cause cell death (85) and lack of selectivity for telomerase has limited this approach. The new nucleotide analogs, 6-methoxy-7-deaza-2'-deoxyguanosine 5'-triphosphate (OMDG-TP) and 6-thio-7-deaza-2'-deoxyguanosine 5'-triphosphate (TDG-TP) inhibited telomerase in cell-free assays with an IC_{50} in the nanomolar range and a quite good selectivity (86)

Specific small-molecule selective enzyme inhibitors, the ideal way to block telomerase activity, do not really exist. The most promising candidate was the non-

nucleosidic, non-competitive inhibitor 2-[(E)-3-naphtalen-2-yl-but-2-enoylamino]-benzoic acid, termed BIBR1532, which was shown to cause telomere shortening and senescence both in vitro and in vivo in mouse xenograft model (87). Recent evaluation of the pharmacological effects showed anyhow, that this kind of compounds exert a direct cytotoxicity by the damage of the telomere structure, thus by causing telomere dysfunction (88). Interestingly the inhibitor did not affect normal cells, which might be a result of a different telomeric structure, or a consequence of the fact that transformed cells possess already shortened telomeres.

Classical antisenses, molecules directed against hTERT mRNA or pre-mRNA, are less established, although emerging knowledge about hTERT function in promoting cancer cell survival independent from telomere elongating activity makes the protein component a promising target. Selected regions are sequences near the 5'-end (77,78), splice sites (89) or computational predicted single-stranded parts (77). Effective ribozyme-constructs targeted the 5'-end (90) or the conserved T-motif (91). siRNAs were also capable to decrease hTERT mRNA and, as a consequence, the protein levels (92). Cell death was often an early event and occurred without telomere shortening (77,89,91) but treatments leading to progressive telomere erosion were as well demonstrated in the literature (93). The reasons for the differences observed in these responses are still unclear. New results, from hTR knock-down experiments using siRNAs (94), indicate that the decrease of telomerase protein level, either through hTERT antisense or through hTR diminution, causes changes in global gene expression, leading subsequently to a rapid cellular response, in contrast to oligomer-based approaches which result in inhibition of telomerase activity and a telomere length dependent delayed effect.

hTERT mRNA pre-mRNA	<ul style="list-style-type: none"> ➤ antisense oligonucleotides (DNA_{PD/PS}) (DNA_{PS}) (2-OMe-RNA_{PS}) (PNA) ➤ ribozyme ➤ siRNA 	(77) (78) (89) (79) (90) (91) (92)
hTR	<ul style="list-style-type: none"> ➤ „antisense” oligonucleotides <ul style="list-style-type: none"> ▪ template region (DNA_{PS}) (2-OMe-RNA_{PS/PD}) (2'-OMOE RNA_{PS}) (PNA) (NP) (NPS) (siRNA) ▪ non-template region (2-OMe-RNA) (2-5A-anti-hTR) (RiAS) ➤ ribozyme 	(69) (70) (72) (70) (71) (73) (74) (81) (76) (82) (83) (75) (84)
Telomere	<ul style="list-style-type: none"> ➤ G-strand overhang antagonists (PNA) ➤ MT-hTR 	(95) (81)

Table 1.: Oligonucleotide type telomere and telomerase inhibitors

Targeting telomeres

Single-strand overhang provides also a target for sequence-specific strategies. Small, 13-mer, PNAs complementary to the G-rich telomeric DNA-strand could induce telomere shortening, cell growth arrest, and apoptosis in AT-SV1 transformed human fibroblasts (95).

Development of G-quadruplex interactive compounds was initiated by the fact that the RNA template region of telomerase requires a linear, non folded telomeric DNA as primer for telomere extension. According to this, presence of telomeric G-quadruplex structure would result in telomerase inhibition. Classes of G-quadruplex inhibitors described to date include anthraquinones, fluorenones, acridines (like BRACO19, RHPS4) cationic porphyrines (e.g. TMPyP4), triazines (such as 12459), 2,6-pyridine-dicarboxamide derivatives (96), indolo-quinolines and the microbial product

telomestatin (SOT-095). The development of these molecules was severely impeded by the relatively poor selectivity for binding to quadruplex versus duplex DNA, however new generation of derivatives has improved potency for telomerase inhibition. G-quadruplex interactive compounds confer their biological activity through multiple mechanisms: first, by interaction with the telomeric G-rich single-strand overhang and thereby affecting telomere capping leading to rapid senescence or apoptosis, and second by inhibiting the access of telomerase to telomeres. Main advantage of a rapid telomere dysfunction is that the onset of the antitumor effect is generally more rapid, than with agents solely inhibiting telomerase activity. Detailed evaluation of the agents' cellular effects showed anyhow, that the explanations might be even more complicated. In cells, treated with 12459 the decrease of telomerase activity was caused by an alteration of the hTERT splicing pattern: an almost complete disappearance of the active ($\alpha\beta$ +) transcript and an overexpression of the inactive β -transcript, caused by the stabilization of quadruplexes located in the hTERT intron 6 (97). Despite of many existing compounds, only a few like BRACO19 (98) could progress into in vivo tests. These agents could theoretically target the telomeres of ALT and normal cells as well.

An interesting approach, using the knowledge about the sequence specificity of telomere function, is the introduction of mutant template telomerase RNA (MT-hTR). Since terminal mutant telomere repeats impair the binding of telomeric proteins and disrupt normal telomeric structure, MT-hTR incorporation into telomerase holoenzyme could cause cell death independent from telomere shortening, so from initial telomere length, overriding the problem of the late onset of the therapeutic effect. Using lentiviral delivery and expression, MT-hTR could be overexpressed in tumor cell lines, enough to compete efficiently with the exceptionally stable endogenous hTR for incorporation into the ribonucleoprotein complex, and cause consequent growth inhibition and apoptosis *in*

vitro and in mouse xenografts (99).

Telomere homeostasis can be influenced as well by targeting the members of the telomere binding protein complexes. As a good example inhibition of tankyrase using PARP-inhibitors has been shown to be effective in concomitant treatment with telomerase inhibitors (100).

Pharmacological regulation of other regulation steps is less evaluated. Trials were made to influence transcription of hTERT by interfering with the hormonal regulation pathways or by using biological response modifiers known to induce differentiation (retinoids/ vitaminD₃), thus hTERT downregulation.

Inhibitors of Protein kinase C or chaperone Hsp90 were also tested to inhibit correct posttranslational processing of telomerase holoenzyme.

TELOMERASE ACTIVATORS

For a very long time research focused just on the strategies to inhibit telomerase. Longevity in the western-type populations turned the attention on chronic diseases and their possible therapy. Activation of telomerase has a potential for the treatment of a broad range of degenerative diseases or chronic conditions in which cellular aging plays a role. New molecules patented by Geron, GRN139951 and GRN140665, were shown to stimulate T-cell proliferation and IFN(γ) production. These compounds are promising for the future integration into the clinical protocols for diseases associated with immune aging, for e.g. AIDS.

ACUTE PROMYELOCYTIC LEUKEMIA

Acute promyelocytic leukemia (APL), a subtype of acute myeloid leukemia (FAB classification: M3) is characterized by the accumulation of abnormal promyelocytes, disseminated intravascular coagulation and the chromosomal translocation t(15;17) which fuses retinoid receptor-alpha (RAR α , located on chromosome 17) to the promyelocytic leukemia gene (PML) on chromosome 15 and leads to the expression of the PML-RAR α fusion protein (101).

Role of retinoids and their receptors in APL

RAR α , a nuclear hormone receptor, binds as RAR/RXR (retinoid X receptor) heterodimer to specific retinoic acid responsive elements (RARE) in the gene promoters and regulate gene expression via transcriptional coactivator and corepressor complexes. In the absence of the ligands the heterodimer binds corepressors, like N-CoR (nuclear hormone receptor-corepressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptors), which recruit histone deacetylases (HDAC) and make inhibitory contacts that interfere with transcriptional initiation. In the presence of ligands, the receptor will release the corepressor-komplex and bind coactivators, like histone covalent modifiers, including acetylases and methylases, ATP-dependent chromatin-remodeling complexes and components of the mediator complexes (like TRAP/DRIP) that assist in the preinitiation complex assembly. RAR α can bind all-trans and 9-cis retinoic acids, but only the all-trans form can induce corepressor release (102).

The PML-RAR α aberrant transcription factor retains the DNA-binding domain and the ligand binding domain, which has an affinity to ATRA comparable to the wild type (103). It binds to the target genes in the form of oligomers or in complexes with RXR (104), recruits N-CoR-HDAC (105) and DNA-methyltransferases, leading to deregulation of retinoic acid target genes, that play critical roles in myeloid differentiation (Fig. 4). As a result maturation is blocked in the promyelocytic stage. Aside from recruiting chromatin modifiers, PML-RAR α seems to exert its oncogenic transcription control by multiple repressive pathways since the unliganded PML-RAR α was shown to interfere with the formation of preinitiation complex (106) even if chromatin dependent repression is released by using HDAC-inhibitors. Affected pathways due to altered transcription will include those using AP1 transcription factor or interferon regulatory factors (107). Beside the above effects it will sequester RXR and PML, latter leading to nuclear body reorganization (108) and decrease of apoptosis (109). Physiological doses of retinoids are insufficient to trigger the release of the N-CoR-HDAC, but pharmacological doses (10^{-6} M) can induce the coactivator-corepressor change, although the recruitment of coactivators and transcriptional apparatus will be still impaired. PML-RAR α -RXR oligomers can be functional targeted by cAMP, which boosts transcriptional activation by ATRA, and restores ATRA triggered differentiation in retinoid-resistant APL cells (110).

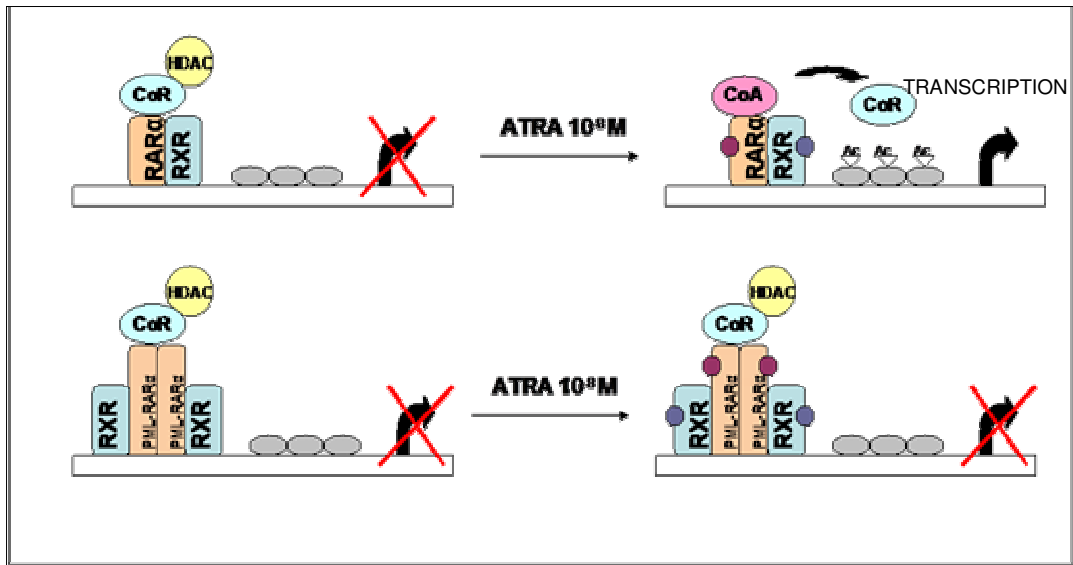


Figure 4.: Leukemogenic effect of PML-RAR α

In the absence of ligand, RAR α /RXR heterodimers recruit transcription corepressors (CoR), which mediate transcriptional silencing by mechanisms that include direct inhibition of the basal transcription machinery and recruitment of chromatin-modifying enzymes. In the presence of physiological concentrations ($10^{-8}M$) of ATRA, the transcription corepressor is released and the coactivator is recruited to the RAR α /RXR heterodimer, resulting in histone acetylation (Ac) and overcoming of the transcription blockage.

PML-RAR α fusion protein binds to target genes either on its own or with RXR and then recruits corepressors, leading to transcriptional repression and myeloid differentiation inhibition.

ATRA-based therapy for acute promyelocytic leukemia

The introduction of ATRA-based differentiation therapy changed not only the prognosis of acute promyelocytic leukemia: it represents the success of biological response modifiers in cancer therapy. Disadvantage of ATRA monotherapy is the relative quick development of resistance. Mechanisms responsible for acquired ATRA

resistance are increased activity of the cytochrome P450 (CYP) enzymes, leading to increased metabolism, upregulation of small cellular RA-binding proteins causing sequestration of ATRA and alterations in the cellular signal acceptor structures, like mutations in PML-RAR α (111). The most dangerous side effects during ATRA induction therapy is the so-called “Retinoic Acid-syndrome” (RAS) in 5-20% of the patients. This syndrome is characterized by respiratory distress, pleural effusions, pericarditis, pulmonary infiltrates, fluid retention, hypotension and fever, typically starting at day 10 of the treatment. Proposed reasons are the release of cytokines, including interleukin-1 β , IL-6, IL-8, and TNF α , by APL cells undergoing differentiation and cell-endothel adhesion followed by extravasation. ATRA also leads to aggregation of APL cells, which appears to be mediated by ATRA induced expression of leukocyte function-associated antigen-1 (LFA-1) and its ligands on the cell surface like ICAM-2. Recommended treatment and prevention of RAS is administration of dexamethasone from the earliest sign until the complete disappearance of the symptoms (112).

Arsenic trioxide in hematologic malignancies

Arsenic trioxide has become a center of attention as it turned out to be the active component in the traditional Chinese receipt Ailing-1, that has been successfully used to induce complete remission in APL patients. First studies showed efficacy of As₂O₃ as single-agent therapy in relapsed or refractory APL after ATRA and anthracycline treatment- the initial multicenter trial, followed by several others, reported a 85% CR rate and molecular remission in most of the patients (113). The use of arsenic trioxide in patients with newly diagnosed disease yielded results similar to those observed in patients with relapsed disease (114). Considering that ATRA and arsenic showed synergistic effect *in vitro* (115) and no cross resistance in clinics, made the idea of

combinational treatment attractive. First experiments confirmed, that combinational induction therapy results in higher rate of molecular remission (116) without increasing toxicity. Despite of encouraging trials, the success of the ATRA plus chemotherapy based induction pushes other protocols into the second line (117). Only patients with comorbid conditions, like sever organ failure or anticoagulant therapy are induced with ATRA/As₂O₃ combination. Side effect profile of arsenic treatment is favorable: gastrointestinal discomfort, reversible neuropathy, transient liver dysfunction and QT-prolongation leading in few cases to torsade de pointes ventricular arrhythmia (118).

Several molecular mechanisms have been implicated in arsenic action. One of the most intensively studied effects is the induction of reactive oxygen species, which could oxidize the cellular proteins leading to cell death. In multiple myeloma, mechanisms involve inhibition of NF- κ B, in chronic myeloid leukemia downregulation of disease-specific Bcr-Abl tyrosine kinase was observed, whereas in solid tumors disruption of angiogenesis as made responsible for the favorable effects.

The palette of mechanisms underlying the cooperative action of ATRA and As₂O₃ on molecular level contains several members. ATRA was shown to induce cleavage of the PML-RAR α fusion protein by caspases (119), as well as proteosomal degradation (120). In contrast to ATRA, which targets the RAR α moiety, arsenic targets the PML moiety of PML-RAR α , through a still unclear mechanism, and causes PML to localize to the nuclear matrix and become sumoylated. Sumoylation is necessary for proteasome recruitment and arsenic-trioxide-induced degradation. Another mechanism underlying the convergence is the phosphorylation of RXR by JNK activated by As₂O₃ (121). Increased phosphorylation of SMRT and its dissociation from the fusion protein was also observed after arsenic treatment (122). Importantly, these effects are not restricted to cells bearing PML-RAR α .

OBJECTIVES

Previous studies have shown that anti-template phosphorothioate-modified oligonucleotides show competitive behavior regarding primer-substrates, suggesting their interaction with a protein motif, termed primer binding site, which normally binds to DNA to be elongated (123)

We intended to design, synthesize and study the *in vitro* activity of oligonucleotide-based telomerase inhibitors which contain, beside the well established anti-template sequence, a chemically modified oligonucleotide moiety known to interact with reverse transcriptases, thus presumably also with the catalytic subunit of telomerase.

- 1) We planed to synthesize antisense (13mer) oligonucleotides carrying $(s^4dU)_n$ ($n = 4, 8, 16, 24$) at its 3' or 5' end, determine the IC_{50} s of these inhibitors on partially purified human telomerase and examine their specificity by measuring their inhibitory activity on HIV reverse transcriptase.
- 2) We wanted to determine the cellular and nuclear uptake of these telomerase inhibitors with confocal laser scanning microscopy studies using fluorescently labeled derivatives of the inhibitors with the most favorable pharmacological properties.
- 3) With the most effective internalization method found during the uptake studies, we would have liked to test the chimeric oligonucleotides on immortal cell lines, to investigate whether they are effective in causing telomerase dependent cell death.

Clinical treatment of acute promyelocytic leukemia (APL) is based on retinoids. All-trans-retinoic acid (ATRA) has also been shown to downregulate telomerase independently from its activity to induce differentiation, thus our interest is turned to potential combinations of ATRA with other types of telomerase inhibitors. Experiments were focused on putative cooperating effect of As₂O₃ and ATRA, as the benefits of sequential or combinational therapy has already been shown.

1) We wanted to demonstrate *in vitro* that the success ATRA/As₂O₃ treatment in APL pathology can be explained at least in part by a synergistic effect of these two drugs in triggering downregulation of telomerase.

2) Our aim was to investigate whether the decrease is efficient enough to cause telomere shortening and subsequent cell death in several ATRA-maturation resistant APL cells.

3) Previous investigations restricted the mechanism of the synergism of ATRA/As₂O₃ to the modulation and/or degradation of PML-RAR α oncoprotein through distinct pathways. Since these events seem not to be important in case of telomerase, determination of the factors and the mechanisms responsible for the repression of the telomerase by the retinoids would be important, to find other points of possible convergence.

MATERIALS AND METHODS

Partial purification of human telomerase

The partially purified human telomerase was prepared from S100 extract of HL-60 cells with slight modification of the previously described protocol (124). Cells were collected and washed in ice cold PBS. The pellet was resuspended in 2.3x Hypo buffer [1x Hypo buffer: 10 mM Hepes-KOH (pH 8.0), 3 mM KCl, 1 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM PMSF, 10 U/ml RNasin (Promega)], incubated on ice for 10 min, then homogenized in a glass homogenizer. After further 30 minutes on ice followed by a centrifugation (10 min, 16 000g) the supernatant was supplemented with one fifteenth volume of 5 M NaCl and centrifuged for 1h at 100 000g at 4°C. This extract was precipitated with 40% ammonium sulfate and the precipitate was collected by centrifugation (10 min, 16 000g). Pellet was resuspended in 1x Hypo buffer supplemented with 10% glycerol, and the solution was applied to DEAE-Sepharose column (Pharmacia). The column was washed with 1x Hypo buffer containing 0.35 M NaCl. Active fractions were pooled and dialyzed against 1x Hypo buffer. Following dialysis, extract was reapplied to DEAE-Sepharose column and eluted as previously. Telomerase inhibition studies with the pooled extract after the first anion-exchange chromatography step or active fractions after final purification gave the same results. Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories).

Oligonucleotides

Chemically modified oligonucleotides used for inhibition studies, Cy5-labeled molecules and primers used in TP-TRAP assay were synthesized and purified in our laboratory (124,125). Briefly, oligonucleotides were synthesized by standard phosphoramidite chemistry on a Gene Assembler Plus (Pharmacia) oligonucleotide synthesizer, and then purified on an ÄKTA Purifier (Amersham-Pharmacia) using Resource Q anion exchange column with 5 mM Tris-HClO₄ (pH 8.2) buffered NaClO₄ eluent gradient (125). For the preparation of chimeras the starting material was an oligonucleotide in which the (s⁴dU)_n part was replaced with (dC)_n then converted to (s⁴dU)_n by H₂S treatment (10 days at 55°C) (126). The same thiolation method was used to prepare (s⁴dU)_n from (dC)_n.

3'-blocked (s⁴dU)₈AS was synthesized using 3'-Spacer C3 CPG (Glen Research, Sterling, Virginia).

Telomerase activity measurement

In the case of kinetic inhibition studies, telomerase activity was measured using a modification of the TP-TRAP (Two Primer-Telomere Repeat Amplification Protocol) assay (124). Briefly, inhibitor oligonucleotides were preincubated at 25°C with the partially purified telomerase enzyme in TRAP-buffer [20 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 63 mM KCl, 0.005% Tween 20, 1 mM EGTA, 0.1 mg/ml BSA], then the enzyme reaction was initiated by the addition of the MTS [AGCATCCGTCGAGCAGAGTT] substrate primer (25 pmol) as well as dATP, dCTP, dGTP (50 μM), dTTP (2 μM), 0.5 μCi [Me-³H]dTTP (Amersham), 2.25 U JumpStart Taq (Sigma) and reverse primers [TAGAGCACAGCCTGTCCGTG and TAGAGCACAGCCTGTCCGTG(CTAACC)₃].

For telomerase mediated extension of MTS (Modified Telomerase Substrate) primer, reaction mixtures (50 µl each) were incubated at 30°C for 10 minutes, then heated to 95°C for 2 min to denature telomerase and activate the JumpStart Taq for hot-start PCR in order to amplify extension products (cycles 1-2: 94°C/30s, 50°C/60s, 72°C/90s; cycles 3-27: 94°C/30s, 63°C/30s, 72°C/30s). After PCR, the products were precipitated with 50 µl 10% TCA containing 0.1 M sodium pyrophosphate, kept on ice for 10 min, then collected by filtration on Whatman GF/C filters. Filters were washed with 5% TCA followed by ethanol, then air dried. ³H-labeled nucleotide incorporation was quantified by liquid scintillation counting. The oligonucleotides for inhibition studies were prepared in various concentrations at a range between 2 nM to 4 µM (final concentrations). Measurements were performed at least as duplicates. Activity was plotted as a function of concentration of the added inhibitor using GraphPad Prism 2.01 software, and IC₅₀ parameters were determined graphically from these graphs, by the software.

Experiments with protein extracts of the oligomer and ATRA/As₂O₃ treated cell cultures were performed with TRAPeze Elisa telomerase detection kit (Qbiogene, Illkirch, France) according to the manufacturer's instruction. The main principle of this enzyme linked immunosorbent assay is similar to that of the TP-TRAP-assay introduced above, as it is based on the PCR-amplification of the telomeric extension products, and the subsequent detection of the labeled DNA pieces. The telomerase mediated extension and PCR-amplification is performed with a biotinylated substrate primer and dinitrophenyl(DNP)-labelled dCTP. The TRAP-products, tagged with biotin and DNP residues, are immobilized onto streptavidin-coated microtiter plates via biotin-streptavidin interaction, and then detected by anti-DNP-antibody, conjugated to horseradish peroxidase. By detecting HRP activity, using substrates 3,3',5,5' -

tetramethylbenzidine and subsequent color development, we can determine the quantity of the products, thus the relative activity of the telomerase.

The above two methods, used to detect telomerase activity changes after treating partially purified telomerase or cell cultures with pharmacons, give similar results, thus can be equally used to monitor inhibitors.

Reverse Transcriptase-assay

Measurement of reverse transcriptase activity was performed as described earlier (126). Inhibitors were preincubated at 25°C for 10 min with 0.1 U HIV reverse transcriptase (Amersham) in a reaction mixture containing 100 mM Tris-HCl (pH 8.0), 50 mM KCl, 6 mM Mg₂Cl, 100µg/ml bovine serum albumin, 5 mM dithiothreitol and 10µM [Me-³H]dTTP. Reverse transcriptase action was primed by adding the template primer [poly(A)·(dT)₁₆], followed by incubation at 37°C for 1 h and terminated with 100 µl 10% TCA containing 0.1 M sodium pyrophosphate. Radioactive products were separated and measured as in the TP-TRAP-assay.

Oligonucleotide uptake studies

A431 human planocellular carcinoma and 293T human emrional kidney cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, from PAA laboratories), 2 mM glutamine (Sigma) and 20 U/ml penicillin - 20 µg/ml streptomycin (Sigma). Cells were plated in 8-well slide chambers and were allowed to adhere overnight, then washed with DMEM without serum and antibiotics, and transfected with Oligofectamine Reagent (Invitrogen) and Cy5-labeled oligonucleotides [Cy5-X₄AS and Cy5-X₈AS] according to the manufacturer's directions. The final concentration of the oligonucleotides in the

transfection mixture was 200 nM. After 4h of incubation, transfection medium was removed and cells were washed with PBS. Following a fixation/permeabilisation step using 4% formaldehyde and 0.2% Triton X-100 in PBS, cells were incubated (15 min at room temperature) with 30 µg/ml Alexa Fluor 488-tagged 528 monoclonal antibodies against epidermal growth factor receptors (EGFR). Cells were then washed and stained with ethidium bromide (0.5 µg/ml) followed by another washing step with PBS. The cellular distribution of Cy5-labeled oligonucleotides in A431 cells was studied by confocal laser scanning microscopy (Zeiss LSM 510). The contours of the plasma membrane and the nucleus were visualized by fluorescent anti-EGFR antibodies and ethidium bromide as described above. For the excitation of Alexa Fluor 488 the 488 nm line of an Ar ion laser, for ethidium a 543 nm HeNe laser and for Cy5 a 633 nm HeNe laser were used. Fluorescence emission was detected through 500-500 nm and 560-610 nm bandpass and 650 nm longpass filters, respectively. With a Plan-Apochromat 63× oil immersion objective (NA 1.4, Zeiss) image stacks of 0.6 µm thick optical sections were collected.

Long-term treatment of cell cultures with chimeric oligonucleotides

293T, human embryonic kidney, cells carrying SV40 T antigen, were treated with 1-3 µM backbone-modified X₈AS, containing two terminal phosphorothioate internucleotide links [X₈AS_(2PS)] in the presence of Oligofectamine. Oligofections were performed as in case of uptake studies, but after 4h of transfection DMEM with 20% FCS was added to have a culture medium with 10% FCS end-concentration without the removal of the inhibitor. Cultures were counted (using Coulter counter, Beckman), replated and transfected every second or third day. Parallel passages were performed on cells receiving only X₈AS_(2PS) or Oligofectamine treatments. Cell proliferation was

expressed as population doubling. Telomerase activity during treatment was monitored from samples collected after 24 and 72h. To control the specificity of X₈AS_(2PS) on the telomerase activity of the cell cultures, X₈SCR_(2PS), containing a scrambled sequence instead of the hTR antisense, was similarly transfected with or without Oligofectamine, and cells collected after 24h were subjected to TRAP-assay.

Cytochemical staining for senescence activated β-galactosidase

Cells were washed in PBS, fixed for 3-5 min in 2% formaldehyde/0.2% glutaraldehyde, washed, and incubated at 37°C (no CO₂) overnight with fresh senescence-associated-galactosidase stain solution: 1 mg of X-Gal DMSO, 40 mM citric acid/sodium phosphate (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl₂. To detect lysosomal β-galactosidase citric acid/sodium phosphate (pH 4.0) was used (127).

Acute promyelocytic leukemia cell-lines

The maturation-resistant human promyelocytic leukemia cell lines NB4-LR1 and NB4-LR2 (128,129) the selected subline NB4-LR1^{SFD} (Saved From Death) (130), the retrovirally infected NB4-LR1/hTERT-GFP (131) and the NB4-LR2/hTERT-GFP sublines expressing both hTERT protein and the green fluorescent protein (GFP) reporter from the same transcript, the NB4-LR1/GFP and the NB4-LR2/GFP sublines expressing only the GFP control vector were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS, from PAA laboratories), 2 mM glutamine (Gibco) and 50 U/ml penicillin - 50 µg/ml streptomycin (Gibco) in a humidified 5% CO₂ atmosphere. We verified that expression of GFP alone did not modify the proliferation and molecular responses of the parental cells to treatment.

In long-term treatments, cells were seeded at 1×10^5 /ml density, and drugs were added to yield the appropriate end-concentration: $1 \mu\text{M}$ for ATRA (Sigma), $0.2 \mu\text{M}$ for As_2O_3 (Sigma) respectively. Every second or third day, cells were counted (Coulter Counter), and reseeded in cell culture medium containing the respective pharmacons.

Quantitative RT-PCR to detect mRNA yielding functionally active telomerase

We detected hTERT transcripts, that give rise to active hTERT protein, with the help of LightCycler TeloTTAGGG hTERT Quantification kit (Roche Diagnostics) using LightCycler Instrument. We amplify a 198 bp fragment with the help of hybridisation probes, labeled with fluorescein or LightCycler Red 640. As a reference and control for RT-PCR performance porphobilinogen deaminase is processed.

Measurement of Terminal Restriction Fragment length

Average telomere length of the treated cells was measured with the Terminal Restriction Fragment (TRF) method using the chemiluminescent TeloTTAGGG Telomere Length Assay (Roche Diagnostics). Genomic DNA was purified with salting out method (132). Cells were resuspended TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) with 2% SDS and digested with RNase A (Sigma) for 30 min at room temperature. Extracts were complemented with NaCl (to end-concentration 0.4 M) and digested overnight with proteinase K (Sigma). After digestion was complete, saturated NaCl was added, the precipitated proteins were separated by centrifugation and the supernatant containing the DNA was transferred to another tube and precipitated with 2 volumes of absolute ethanol. The precipitated strands were removed with a pipette, dried and resuspended in TE. $3 \mu\text{g}$ of the purified DNA was digested with the restriction enzymes Hinf I and Rsa I. These frequently cutting enzymes have no recognition site in

the telomeric sequences, thus leave them intact in contrast to the non-telomeric DNA, which is degraded in small fragments. Digested DNA was then separated by gel electrophoresis, and transferred to positively charged nylon membrane (Roche) by traditional capillary Southern blot. Blotted sequences are hybridized with digoxigenin-labeled probe complementary to telomeric repeats, incubated with an anti-digoxigenin antibody coupled to alkaline phosphatase, and visualised by using a chemiluminescent substrate of alkaline phosphatase. A population of cells provides the average telomere length of the sample, indicated by a smear and we can estimate the TRF-length by comparing the mean size of the smear to a molecular weight marker.

Real-time quantitative PCR for hTERT expression after AzaC and ATRA

NB4-LR1 cells were plated and treated with 0.25-0.5 μ M 5-azacytidine (AzaC). On day 6 the cultures were divided, and half of the cells were treated with 1 μ M ATRA in addition, while the other half was cultured just as before. The samples were collected on day 4 of the ATRA treatment. RNA was isolated with TRIzol Reagent (Invitrogen), according to the manufacturer's instruction. The purified RNA was digested with RQ1 RNase-Free Dnase (Promega) to eliminate DNA contamination. Reverse transcription was performed at 42°C for 30 min from 100 ng of total RNA using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega). Quantitative PCR was performed using real-time PCR (ABI PRISM 7900, Applied Biosystems), 40 cycles of 95°C for 12 s and 60°C for 1 min using Taqman assays. All PCR reactions were done in triplicate with one control reaction containing no RT enzyme. Absolute standard curve method was used to quantify transcripts and normalize for cyclophilin. Taqman-assay for hTERT was performed with the primers 5'-TTCCTGCACTGGCTGATGAG-3' (fwd),

5'-TCTTTTGAAACGTGGTCTCCG-3'(rev) and FAM-TAMRA-labeled probe
5'-ACGTCGTCGAGCTGCTCAGGTCTTTC-3', corresponding to a region included in
all splice variants (FAST DB, 133).

For hCyclophilin we used the following primers and probes:

5'-ACGGCGAGCCCTTGG-3'(fwd), 5'-TTTCTGCTGTCTTTGGGACCT-3'(rev),
5'-CGCGTCTCCTTTGAGCTGTTTGCA-3'(probe).

Chromatin Immunoprecipitation Assay (ChIP)

ChIP assays were performed using the Chromatin Immunoprecipitation (ChIP) Assay Kit from Upstate, and following the instructions of the kit supplier. Briefly, APL cells ($\approx 1 \times 10^7$) receiving ATRA (1 μ M), As₂O₃ (0.2 μ M), 8-(4-chlorophenylthio)adenosine cyclic 3'5'-monophosphate (CPT-cAMP, 200 μ M) treatment in different combinations were fixed with formaldehyde (final concentration 1% v/v) in culture medium at 37°C for 10 min. Fixed cells were pelleted by centrifugation and washed twice in ice cold PBS. The cells were then resuspended in SDS lysis buffer supplemented with Protease Inhibitor Cocktail (Sigma), kept on ice for 20 min and were sonicated (Branson sonicator, five times with 10 sec pulses and 50 sec rest between each pulse) to make soluble chromatin. After centrifugation, samples were diluted 10-fold dilution with ChIP dilution buffer; aliquots of total chromatin were taken at this point to use as a positive control in the PCR. The cell lysates were precleared by incubation with Salmon Sperm DNA/Protein A agarose Slurry (50%) and then immunoprecipitated with anti-AcH3 or anti-AcH4 (Upstate Biotechnology) antibodies at 4°C overnight. Antibody/histone complexes were collected by incubation with Salmon Sperm DNA/Protein A agarose Slurry. Beads were subjected to several rounds of washing using the following buffers:

Low Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl),

High Salt Immune Complex Wash Buffer, (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500 mM NaCl),

LiCl Immune Complex Wash Buffer, (0.25 M LiCl, 0.5% NP-40, 0.5% deoxycholic acid, 20 mM Tris, pH 8.1) and

TE Buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8).

Bound DNA-protein complexes were eluted from the antibodies with two incubations in elution buffer (0.1 M NaHCO₃, 1% SDS) at room temperature for 15 min. Cross-links were reversed by incubation at 65°C for 4h and the sequential proteinase K-digestion (45°C, 1h). DNA was extracted with QIAquick PCR Purification Kit (Qiagen). Products from the CHIP assay were amplified using the primers 5'-AACAGATTTGGGGTGGTTTG-3' (sense), 5'-CTGGCCTGATCCGGAGAC-3' (antisense) flanking the region -712/-435 of the hTERT promoter. PCR cycles used: 94°C/10min, then 33 cycles - 95°C/30sec, 54°C/45sec, 72°C/45sec and finally 72°C/5min. As a positive control a fragment of the RAR β gene promoter was amplified using the primers 5'-TCCTGGGAGTTGGTGATGTCAG-3' (sense) and 5'-AAACCCTGCTCGGATCGCTC-3' (antisense). PCR conditions were as follows: 94°C/5min for denaturation, followed by 33 cycles - 95°C/30sec, 58°C/1min, 72°C/1min. PCR products were analyzed on 2% agarose gels stained with ethidium bromide. An aliquot of total chromatin was subjected to PCR analysis as input control, as well as no antibody controls to check if background is present. Oligonucleotides were all purchased from Prologo France, PCR was performed with DyNAzyme EXTTM (Finnzymes) with 5% DMSO as additive in case of the GC-rich hTERT sequences.

RESULTS I.

TELOMERASE DEPENDENT CELL DEATH USING CHIMERIC OLIGONUCLEOTIDES AS TELOMERASE INHIBITORS

Effect of 3' or 5' attached (s^4dU)_n on the telomerase inhibiting potential of a 13mer antisense oligonucleotide

The (s^4dU)₃₅ (abbreviated as X₃₅; X represents a 4-thio-deoxyuridylate unit, structure shown on Fig. 5) was a potent competitive inhibitor of HIV reverse transcriptase and human telomerase with respect to the functioning RT template-primer and telomerase substrate primer (124, 126).

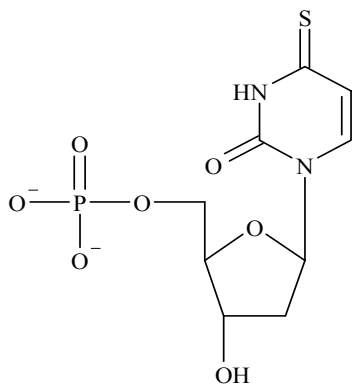


Figure 5.
Structure of the monomer unit of (s^4dU)_n (4-thio-deoxyuridylate).

The inhibitory features of X₃₅ prompted us to develop chimera oligonucleotides, presumably specific inhibitors of telomerase, composed of an antisense sequence and a 3' or 5' X_n (n = 4, 8, 16, 24) moiety, which is thought to interact with the protein subunit of human telomerase. This type of chimeric molecules would fulfill the predicted requirements of targeting both the catalytic and the RNA-component of the enzyme.

First we determined the IC₅₀ values for the inhibitors on partially purified telomerase using the TwoPrimer-TRAP assay.

The telomerase inhibiting potential of X₃₅ was significantly affected by preincubation of the inhibitor with the enzyme (124). Since inhibitory parameters of chimeric molecules were also likely to be affected by the duration of preincubation, first we tested the effect of various preincubation times on inhibition (Fig. 6).

We chose for this experiment the oligonucleotide X₁₆AS in 40 nM concentration, as we knew from preliminary studies that it generates analyzable data in contrast to the utilization of a much weaker or a much stronger inhibitor. Measuring the telomerase activity after enzyme exposure to the inhibitor for 0 to 30 minutes, we could verify that preincubation time is a significant parameter that influences the effect of chimeric molecules. For the IC₅₀ studies we chose 10 min preincubation, because this period was measured to be obligatory and sufficient for the chimeras to exhibit maximal inhibitory potential. Considering the fact that oligonucleotides are relatively large molecules that need to hybridize to the target sequence to act as inhibitors this is not an unusual phenomenon, underlined also by the experimental layout for telomerase inhibition studies of other oligonucleotides (72, 134).

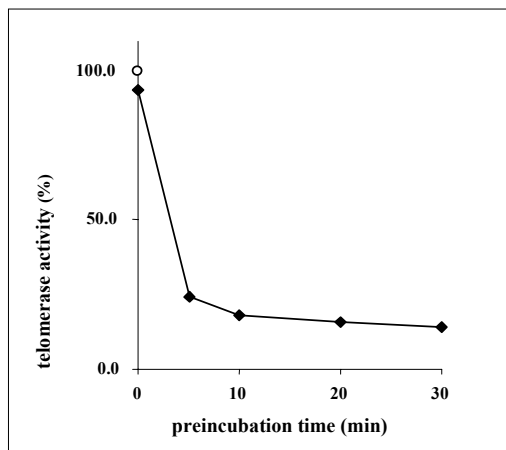


Figure 6.
Effect of preincubation time on the inhibitory activity of X₁₆AS. Black diamonds indicate the telomerase activity measured with TP-TRAP assay in the presence of 40 nM X₁₆AS. Empty circle represents telomerase control without inhibitor.

The 13mer phosphodiester antisense oligonucleotide (AS), designed to target hTR template region, inhibited the partially purified telomerase with an IC₅₀ of 174.2±14.1 nM (Table 2).

Sequence specificity of AS-mediated inhibition was proven by scrambled control (IC₅₀_{SCR} > 4000 nM; Table 2).

The chimeras were prepared by thiolation of the (dC)_n-extended AS. Although thiolation procedure was not expected to cause chemical modification (135) to ensure that the antisense (AS) component remains unaltered, we thiolated a sample of AS, too, according to the standard procedure. It must be noted that the AS 13mer (free or in chimeras) does not contain cytidilates, which would be sensitive to H₂S (for sequences see Table 2). Comparison of the UV-spectra and the inhibition parameters before or after the thiolation showed no difference, in good agreement with the literature (135).

Antisense oligonucleotides with 5' (s⁴dU)_ns were more potent inhibitors either than the antisense alone or the 3' modified ones (Table 2 and Fig. 7 as an example of a dose-response curve).

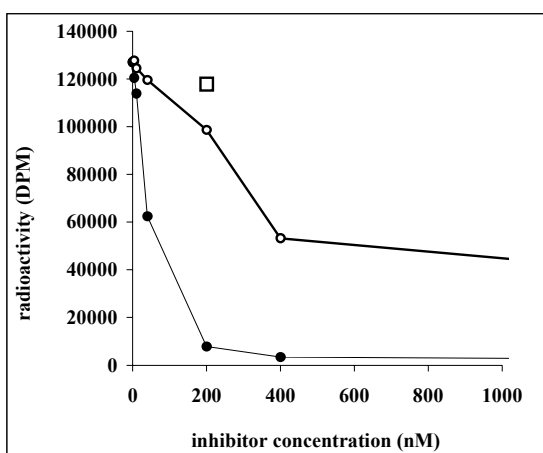


Figure 7.

Dose-response curve for inhibition of telomerase with X₈AS (filled circles) or ASX₈ (empty circles). Enzyme activity (presented as DPM values) was measured by TP-TRAP assay. The square represents control experiments for JumpStart Taq using X₈AS or ASX₈ at 200 nM, added after reaction and before the PCR step.

Increasing length of the thiolated moiety increased the telomerase inhibitory potential up to IC₅₀ = 24 nM, when 16 or 24 modified bases were attached to the 5'-end. However, the inhibitory activity of the 3' X₄ and X₈ modified oligonucleotides were less than that of the 13mer antisense. A further increase of the length of the 3' modified moiety increased the inhibition close to the same extent than the 5' modified counterparts (IC₅₀ [ASX₂₄] = 31.32 nM).

Name	Sequence	IC50±SD(nM)	Name	Sequence	IC50±SD(nM)
AS	AGTTAGGGTTAGA	174.2 ± 14.1	SCR	GTGATGATGATGA	>4000
(X)₄AS	(s ⁴ dU) ₄ AGTTAGGGTTAGA	134.2 ± 16.9	SCR(X)₄	GTGATGATGATGA(s ⁴ dU) ₄	2127.1 ± 509.9
(X)₈AS	(s ⁴ dU) ₈ AGTTAGGGTTAGA	38.2 ± 12.4	SCR(X)₈	GTGATGATGATGA(s ⁴ dU) ₈	154.4 ± 19.6
(X)₁₆AS	(s ⁴ dU) ₁₆ AGTTAGGGTTAGA	24.0 ± 3.6	SCR(X)₁₆	GTGATGATGATGA(s ⁴ dU) ₁₆	65.7 ± 9.2
(X)₂₄AS	(s ⁴ dU) ₂₄ AGTTAGGGTTAGA	24.3 ± 3.6	SCR(X)₂₄	GTGATGATGATGA(s ⁴ dU) ₂₄	25.3 ± 11.3
AS(X)₄	AGTTAGGGTTAGA(s ⁴ dU) ₄	292.4 ± 10.6	(C)₄AS	(C) ₄ AGTTAGGGTTAGA	2760.1 ± 617.4
AS(X)₈	AGTTAGGGTTAGA(s ⁴ dU) ₈	308.1 ± 13.7	(C)₈AS	(C) ₈ AGTTAGGGTTAGA	>4000
AS(X)₁₆	AGTTAGGGTTAGA(s ⁴ dU) ₁₆	112.4 ± 25.5	(C)₁₆AS	(C) ₁₆ AGTTAGGGTTAGA	***
AS(X)₂₄	AGTTAGGGTTAGA(s ⁴ dU) ₂₄	31.3 ± 13.5	(C)₂₄AS	(C) ₂₄ AGTTAGGGTTAGA	417.1 ± 66.2
(X)₄	(s ⁴ dU) ₄	>4000			
(X)₈	(s ⁴ dU) ₈	2974.1 ± 365.6	(X)₈ASsp	(s ⁴ dU) ₈ AGTTAGGGTTAGA spacer	28.3 ± 5.1
(X)₁₆	(s ⁴ dU) ₁₆	310.9 ± 76.0			
(X)₂₄	(s ⁴ dU) ₂₄	116.6 ± 8.1			

***telomerase activity was elevated by C₁₆AS

Table 2. Inhibition of telomerase by oligonucleotides. Shown are synthesized inhibitors with 3' or 5' (s⁴dU)_n and respective controls.

Control experiments for chimeric molecules included oligonucleotides comprising just the chemically modified component $[X_n]$, scrambled molecules with 5' $(s^4dU)_n$ s to prove sequence specificity $[(X)_n\text{SCR}]$ and parent compounds for chemically modified chimeras $[(C)_n\text{AS}]$ to evaluate the role of the thiolated moiety. X_n homooligomers inhibited telomerase in chain length dependent manner, similarly as observed on HIV RT (126), where 25-40mers were shown to be active. Derivates containing scrambled sequences showed weak inhibition when the modified part was short, but the IC_{50} for $X_{24}\text{SCR}$ reached the level of $X_{24}\text{AS}$. 5' cytidilates decreased, almost abolished, anti-telomerase activity of the AS sequence. A surprising phenomenon was observed with $C_{16}\text{AS}$. We found that it substantially increased the telomerase activity. (Experiments were repeated several times with oligonucleotides of different origin.) As this augmentation was only present in the case of $C_{16}\text{AS}$, we suggest that it might be the result of an aptamer-like effect, underlined by the fact that base pairing between cytidilates and the G-rich AS-part is very likely.

Effect of blocking the 3'-OH of $X_n\text{AS}$

The $(X)_n\text{AS}$ oligonucleotide inhibitors carry telomeric sequences at their 3'-end, therefore they can be utilized as telomerase substrates elongated by telomeric repeats. During extension steps the 5' $(X)_n$ moiety could insure the high affinity attachment of the inhibitor substrate to the catalytic subunit.

To evaluate if the extension of the inhibitors has a role in their function, we used oligonucleotides carrying 3' propyl-group as blocker of the 3'-function $[(X)_8\text{ASsp}]$. As demonstrated in Table 2, we did not see a difference between the IC_{50} of $(X)_8\text{AS}$ and $(X)_8\text{ASsp}$ ($IC_{50} [X_8\text{AS}]$: 38.2 nM and $IC_{50} [X_8\text{ASsp}]$: 28.3 nM \pm 5.1).

Specificity of chimeric oligonucleotides for telomerase

Caveats for PCR-based assays like TRAP always include cautiousness in aspect to DNA-polymerase inhibitors, as they could inhibit Taq-polymerase, too. To exclude this possibility control experiments were run with 200 nM of the appropriate inhibitors, which were added in this case after the telomerase reaction, but before the PCR. Amplification was barely affected by the presence of the inhibitors (Fig. 7 and data not shown), indicating that the effects observed in the TP-TRAP-assay were due to the inhibition of telomerase.

Specificity of the oligonucleotides was also proven on HIV reverse transcriptase, which shares common motifs with hTERT (136). We tested all synthesized oligonucleotides in reverse transcriptase assays using them at concentrations that corresponded to the IC₉₀s for telomerase. (4 μM, the maximal tested oligonucleotide concentration in the TP-TRAP-assay, was used in cases when calculated IC₉₀ would have been higher than this concentration.) The conditions were the same as in the TRAP-assay. Chimeras with long modified moieties turned out not to be specific for telomerase (Fig. 8), as suggested by the 90% inhibition of RT at their IC₉₀ for telomerase. In contrast, AS and AS with 4 or 8 (X)s were weak inhibitors of the viral reverse transcriptase.

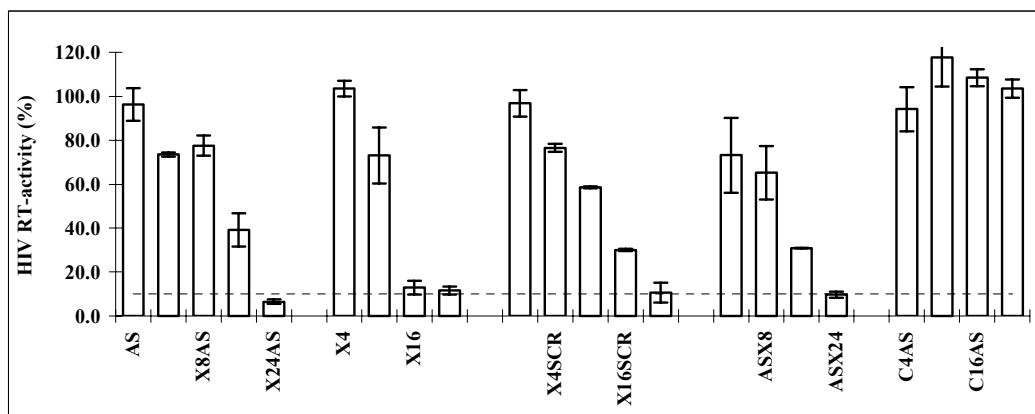


Figure 8. Inhibition of HIV reverse transcriptase by anti-telomerase oligonucleotides.

Applied concentrations correspond to the IC_{90} (dashed line indicates 10% activity) of the inhibitor for partially purified telomerase or $4 \mu M$, the maximal tested concentration in the TP-TRAP assay (in cases when calculated IC_{90} would have been higher than this concentration). RT-activity assays were performed in duplicate, reported values are averages, error bars correspond calculated standard deviations (\pm).

Cellular uptake of oligonucleotide inhibitors

The efficiency of antisense strategies is highly dependent on the cellular delivery of oligonucleotide agents. It was found in our laboratory that X_{35} does not penetrate into the cell (137), suggesting that we may face similar problem with X-extended anti-telomerase oligonucleotides. We examined therefore the cellular uptake of the inhibitors with confocal laser scanning microscopy using Cy5-labeled oligonucleotides. Chosen candidates for uptake studies were X_4AS and X_8AS , since their inhibition characteristics seem to be the most attractive for anti-telomerase strategies in cellular systems.

X₈AS showed significantly improved inhibitory activity on telomerase (IC₅₀ [X₈AS]: 38.2 nM compared to IC₅₀ [AS]: 174.2 nM), sequence specificity (IC₅₀ [X₈AS]/IC₅₀ [X₈SCR]: 38.2 nM/154.4 nM) and a good selectivity (200 nM corresponding to the IC₉₀ for telomerase inhibited RT just by 22.5%). X₄AS is less effective as a telomerase inhibitor (IC₅₀ [X₄AS]: 134.2 nM) but it is favorable because of its high sequence specificity (IC₅₀ [X₄SCR]: 2127.1 nM). Their length and the number of chemically modified nucleotides also supported the possibility of the cellular uptake. We treated A431 human planocellular carcinoma cells with 200 nM Cy5-X₄AS or Cy5-X₈AS and Oligofectamine, a lipid-type carrier. After 4 hours of transfection cells were fixed and stained. Fluorescent-labeled antibody against epidermal growth factor receptor (EGFR) was used to visualize the plasma membrane allowed by the fact that A431 are known to express EGFR in high number (138). Nuclei were stained with ethidium bromide. Inhibitor-transfected cultures showed no morphological changes and no signs of acute toxicity with light microscopy compared with the control samples treated only with Oligofectamine.

Pictures obtained with the confocal microscope clearly showed the internalization of the chemically modified oligonucleotides (Fig. 9). Labeled inhibitors were found in the cytosol and in the nucleus as well, with a characteristic perinuclear enhancement.

Difference between Cy5-X₄AS or Cy5-X₈AS transfections could not be observed. Cell line 293T was also examined (pictures not shown) and showed similar intracellular oligonucleotide distribution.

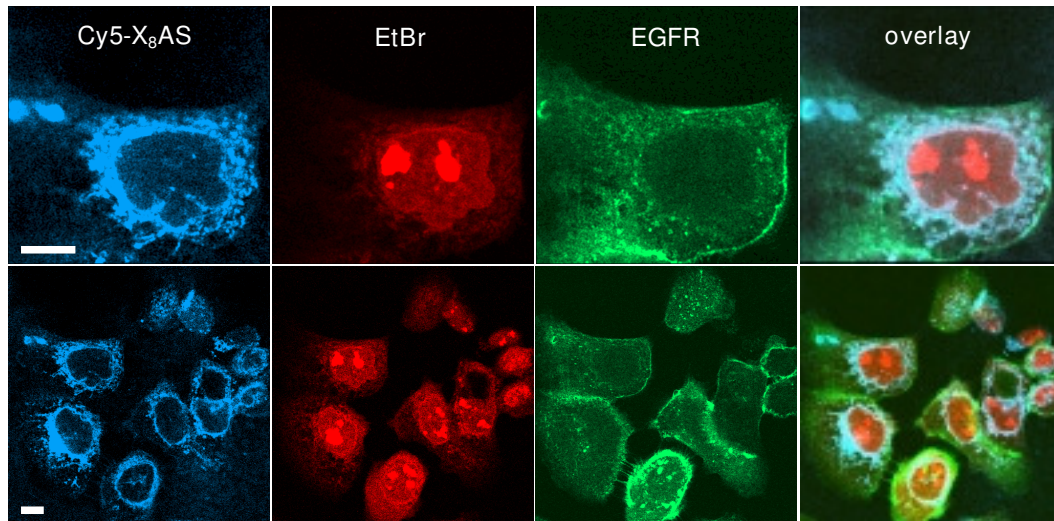


Figure 9. *Optical sections of A431 cells treated with Cy5-tagged X₈AS. Oligonucleotides (Cy5-X₈AS, blue) are present in the cytoplasm and the nucleus as well, showing a perinuclear enrichment. The nucleus was stained by ethidium bromide (EtBr, red). Epidermal growth factor receptors (EGFR) were labeled by Alexa Fluor 488-tagged 528 monoclonal antibodies to visualize the plasma membrane (green). Scale bar: 10 μ m.*

Sustained X₈AS_(2PS) treatment of telomerase positive cells

Inhibition of telomerase activity is not supposed to lead to immediate cell death. Elimination of the telomere maintaining mechanism acts like a permissive event for replication-dependent shortening of the chromosome ends, thus effect of telomerase-specific enzyme inhibitors can be verified just in case of continuous administration of the respective compounds.

We treated a short-telomere variant of 293T cells with 1-3 μM $\text{X}_8\text{AS}_{(2\text{PS})}$, since X_8AS bearing the higher inhibitory potential could be equally internalized as X_4AS . $\text{X}_8\text{AS}_{(2\text{PS})}$ differs from X_8AS , used in the kinetic studies, in two 3' terminal internucleotide bounds which have been changed from phosphodiester to phosphorothioate. This modification has been introduced to enhance inhibitor stability in the cellular environment, without increasing aspecific association known to be a property of fully phosphorothioate oligonucleotides. 24h after treatment telomerase activity was measured to verify the specific action of the inhibitor, also in comparison with $\text{X}_8\text{SCR}_{(2\text{PS})}$. Examined cultures included treatments with oligonucleotides and Oligofectamine Reagent, “oligo only”, as well as untreated controls and samples just with the transfection agent.

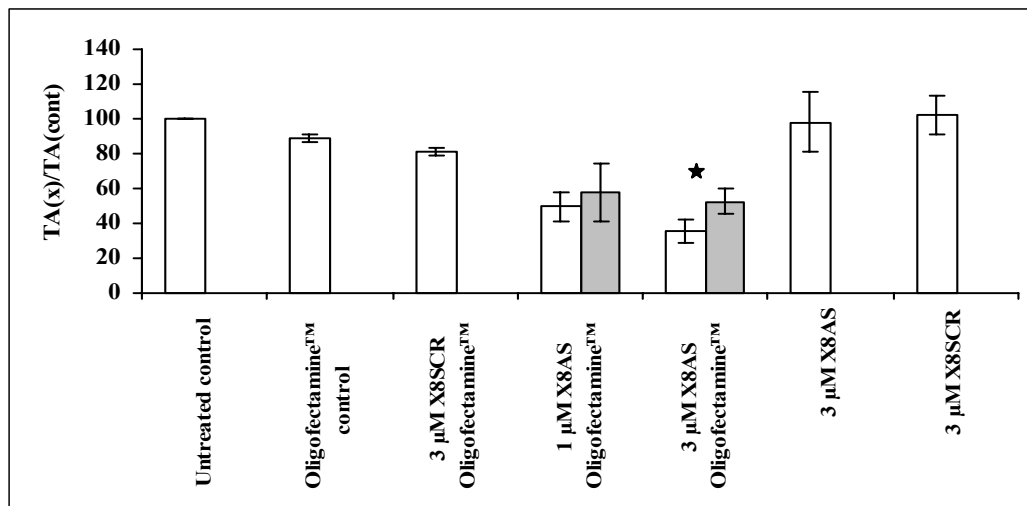


Figure 10. Telomerase activity of oligonucleotide treated 293T cultures 24h (white bars) and 72h (grey bars) after transfection measured with TRAPeze Elisa. Measurements were performed as triplicates, reported are averages and standard deviations. Cell population marked with asterisk underwent cell death after long-term treatment.

Telomerase activity in cultures treated with the antisense containing chimera and its scrambled pair was unchanged. The transfection agent alone caused a diminution of 10%, most probably as a consequence of the nonspecific toxicity the liposomes observed also in decrease of the cell growth and change of the microscopic morphology. Transfected inhibitors could decrease telomerase activity by 75% after 24h, when applied at a 3 μ M concentration. After 72h without re-transfection TA increased to 50% of the untreated control. X₈SCR_(2PS) in similar conditions had just a slight effect: it caused a 10% decrease in addition to Oligofectamine.

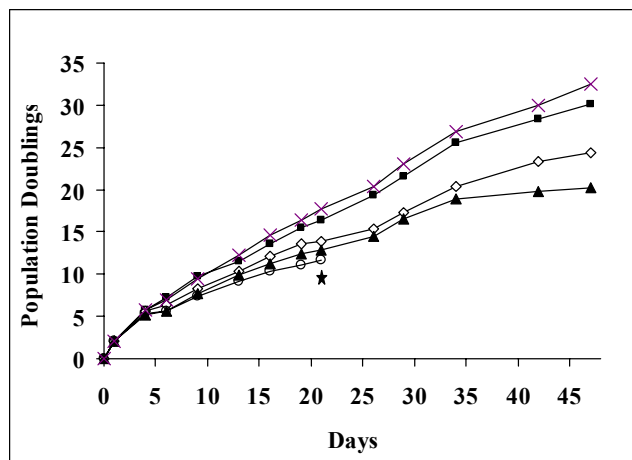


Figure 11.

Proliferation of 293T cells during long-term treatment with X₈AS_(2PS). Empty circle, marked with asterisk represents dying cell population treated with 3 μ M X₈AS_(2PS) and Oligofectamine. Black triangle shows cultures transfected with 1 μ M inhibitor. Controls were

untreated cells (cross), 3 μ M X₈AS_(2PS) “oligo-only” (black square) and cultures treated with Oligofectamine empty diamonds).

Cultures treated with 3 μ M X₈AS_(2PS) in the presence of Oligofectamine died after 20-25 days (Fig 11). The transfection agent alone caused a slight decrease in cell growth compared to the untreated population. A diminution in the same extent could be observed in case of 1 μ M treatment with lipids. Cells with just oligos did not show any kind of change in growth kinetics, as it was predictable after unchanged TA.

DISCUSSION I.

Characteristics of telomerase oligonucleotide interactions

The inhibitory potential of the above presented chimeric oligonucleotides seem to support the dual targeting hypothesis, namely that AS sequence of X_nAS inhibitors forms double helix with the template region of hTR, while X_n interacts with the protein part (Fig. 12) and the two interactions enhance each other. However, structural proves will need detailed experiments. Evaluation of primer-enzyme interactions proved that beside the interaction with the 3' of the primer at the catalytic site, the upstream region of the DNA also has a sequence-specific contact with the protein, which seems to influence binding affinity as well as rate of polymerization (139). It seems to be possible that the protein surface that normally interacts with the 5' of its primer-DNA has a high affinity for thiolated oligonucleotides too, and their binding impairs the catalytic activity or inhibits the interaction of the telomerase with the natural DNA-substrate. The fact that blocking of the 3' end has no effect on inhibition suggests that extension of the chimeric molecules has no crucial role in the mechanism. Reported experiments using phosphorothioate modified telomeric repeats with 3' deoxy-nucleotides gave the same results (113). Of course we cannot exclude that non-blocked inhibitors might be elongated.

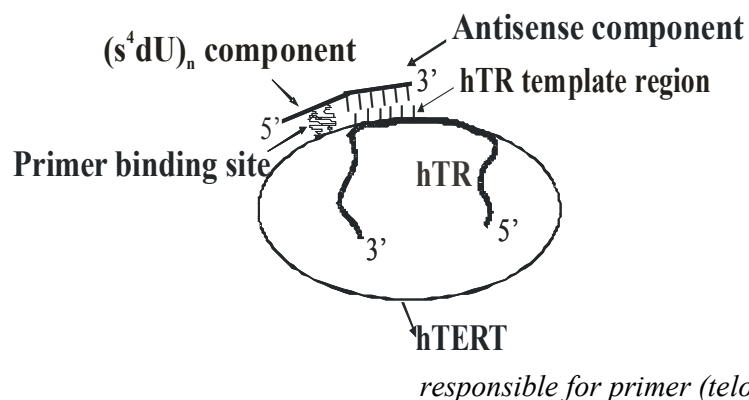


Figure 12.

Putative mechanism of X_nAS inhibitors. AS forms a double helix with the template region of hTR, and X_n interacts with the protein part

responsible for primer (telomerase substrate) binding

In case of ASX_n type of inhibitors, the hybridization to the RNA and the binding of the chemically modified part to the protein (presumably to other sites than 5'-extended ones) is also possible. The “antisense”-contact to the RNA is slightly weakened when short thiolated moiety is attached to the 3'-end, indicating that they may disturb the interaction of the AS with the target sequence. The similar inhibitory activity of X_{24} modified antisenses (3' or 5') indicates that these long inhibitors may not follow the proposed mode of action. This conclusion was also supported by the potent inhibitory potential of the $(X)_{24}SCR$ which is not able to form base pairs with the hTR template sequence (IC_{50} s of $(X)_{24}AS$, $AS(X)_{24}$ and $(X)_{24}SCR$ did not differ essentially). Moreover these derivatives are not specific, since they inhibited HIV RT with similar parameters (see Fig. 8). These findings can be explained by the property of the chemically modified oligonucleotides to inhibit DNA-polymerases in a sequence nonspecific manner, since 37mer chimeras (X_{24} modified antisenses and scramble) reach the length reported to have maximal inhibitory activity [(30 to 40mers) (126)]. In contrast, X_{24} (as shown by its IC_{50} in Table 1) is too short to exert full sequence nonspecific inhibition.

Considering is the physico-chemical nature of the interaction of thiolated oligonucleotides with the protein, there are two likely modes: (a) hydrophobic interaction, due to the hydrophobic character of the 4-thiono group, or (b) disulfide bond formation between properly localized cysteinyl side chains on hTERT surface and reactive $-SH$, formed on the (s^4dU) base due to tautomeric conversion. The latter, covalent interaction between proteins and (s^4dU) containing oligonucleotides is chemically feasible and was proven (137).

Cellular uptake of oligonucleotides

An important factor, proved by the Cy5 labeled chimeras, was the observed nuclear uptake, since telomerase exerts its function as a DNA-polymerase obligatory in the nucleus. The perinuclear portion might correspond to the dissociating lipid-oligonucleotide complexes (140). Experiments to evaluate hTERT subcellular localization in immortal cell lines showed that hTERT is immunologically detectable not only in nuclei but also in the cytoplasm (141). Strong Cy5 signals detected in the cytoplasm may also correspond, at least to some degree, to oligonucleotides bound to the cytoplasmic telomerase. Whether this interaction has a pharmacological significance, needs further investigations.

Chimeric oligonucleotide telomerase inhibitors in immortal cell cultures

Chimeric oligomers were able to decrease the telomerase activity in a dose dependent manner which was sufficient to cause the death of the treated cell population after continuous long-term administration. The delayed onset of the phenotypic effect can be explained by the fact that these inhibitors suppose to decrease the activity of the telomerase, but not the protein levels, thus cell death results from critical telomere shortening after several cell divisions. Although precise nature of the achieved cell death was not evaluated, senescence seems to be excluded, since dying cell population neither showed senescent phenotype nor displayed enhanced senescence activated β -galactosidase activity. T antigen is known to bind p53 protein and cause a perturbation in its function, thus cell carrying this viral oncogene are considered to have a functionally inactive p53 (142). Although opinions are contradictory, it seems that senescence triggered by telomere shortening involves p53 (143), and that p53 status is one of the determining factors in the choice between senescence and apoptotic cell

response in vivo (144). The above factors could explain the absence of senescence as a response on telomere dysfunction in 293T cells.

As clearly seen on the growth curves, cell death occurred just in case the telomerase activity could be kept below a certain level. Since 1 μ M, yielding a 50-60% TA had no effect on cell proliferation, threshold for telomere maintenance seems to be below that level. This reinforces the method of re-transfection after 48-72h, since also in cultures effectively treated [3 μ M X₈AS_(2PS) + Oligofectamine] activity of telomerase after 72h was as high as 50%, due to oligonucleotide degradation and dilution of intracellular concentration as a consequence of cell division. X₈AS_(2PS) and X₈SCR_(2PS) without liposomal agents could not enter the cells as proven by their telomerase activity. Despite of this, they could affect the cellular response, since their binding to proteins localized in or on the membrane is possible, similar to X₃₅ (137). Although we can not exclude this possibility, we could not observe signs of neither acute nor chronic toxicity.

In conclusion, the properties of the novel family of chimeric oligonucleotides indicate that further development of such molecules might be a favorable anti-telomerase strategy, considering additional improvement in inhibitory parameters and intracellular properties as well (for e.g. by introducing various backbone modifications). Further tests on immortal, telomerase positive cell lines of different cellular origin are needed to evaluate susceptible cell lines, possible limiting factors, and pharmacological combination possibilities. Experiments have to be made to validate intracellular molecular events and telomerase-chimera interaction as well. Following the idea of dual interaction, using the presented chemically modified chimeras as lead compounds, could provide new data in the biology of telomerase inhibition.

RESULTS II.

TELOMERASE DEPENDENT CELL DEATH USING ATRA AND As₂O₃

Effect of the sustained ATRA/As₂O₃ combinational treatment on the viability of maturation resistant promyelocytic leukemia cell lines

Majority of therapy-naive acute promyelocytic leukemia cells as well as NB4 APL cell line enters granulocytic differentiation upon ATRA treatment (145). As shown previously, cell death can be achieved also in some cell lines resistant to ATRA-induced differentiation (NB4-LR1), since long-term treatment with pharmacological doses is able to repress telomerase catalytic subunit expression, thus cause death due to telomere shortening (131). Repression of telomerase with subsequent cell death can also be obtained using synthetic RAR α and RXR selective agonists not only in NB4-LR1 but also in “ATRA-unresponsive” NB4-LR2 cells, harbouring a dominant negative, truncated PML/RAR α (146). Subline NB4-LR1^{SFD} retains a functional pathway of telomerase downregulation associated with maturation, but its hTERT expression fails to respond to ATRA or selective agonists (130). Cell death of long-term ATRA-treated NB4-LR1 cells is caused by telomere shortening due to the downregulation of hTERT, however NB4-LR2 and NB4-LR1^{SFD} cells resist to this treatment. Since ATRA as well as As₂O₃ inhibits hTERT expression, we wondered whether this combination, proven to be effective in clinic, could affect synergistically ATRA-resistant cells. It has been previously shown that treatment of NB4-LR1 and NB4-LR2 cells with high concentrations (1 μ M) of As₂O₃ alone or in combination initiates massive apoptosis (115). In these conditions the assessment of a molecular synergy of long-term treatment with ATRA would be difficult. Furthermore, one critical factor that limits the utility of

As₂O₃, especially in case of extended treatments, will be its cytotoxicity to normal tissues, thus, one of our objectives was the substantial reduction of its dose. For this reason NB4-LR1, NB4-LR1^{SFD}, and NB4-LR2 cells were treated with As₂O₃ at low concentration (0.2 μM) in the presence or absence of ATRA (1 μM).

Long-term treatment of NB4-LR1 cells with 1 μM ATRA induced cell growth arrest followed by cell death at day 75, similar to previous reports (Fig. 13A). NB4-LR1^{SFD} and the NB4-LR2 cells continued to proliferate even after 3 or 4 months of continuous treatment with ATRA. 0.2 μM As₂O₃ alone had no effect either on proliferation or viability in none of the cell lines included in the study. Combinational treatments, using both drugs, accelerated cell death in NB4-LR1 compared to the population treated with ATRA alone, and reduced cell growth and viability in both NB4-LR2 cells and NB4-LR1^{SFD}.

Changes in the telomerase homeostasis in the APL cell lines receiving combinational treatment

As previously shown, growth arrest in NB4-LR1 after long-term exposition to ATRA is a consequence of telomerase catalytic subunit downregulation, leading to a marked decrease in telomerase activity, and finally to telomere shortening (131). To evaluate how telomerase is affected by the combinational treatment we measured hTERT expression and TA, as well as changes in telomere length in all three cell lines after extended treatment with ATRA and As₂O₃ alone or as co-treatment. In the ATRA-prone population of each cell line we could observe a remarkable reduction in hTERT mRNA level (Fig. 13B), measured by quantitative RT-PCR. However, only in the NB4-LR1 cells was this decrease (95%) associated with an efficient diminution of TA (Fig. 13C) and telomere shortening (Fig. 14A) resulting in cell death (as previously seen on

Fig. 13A). In these cases, no telomere shortening was observed (Figure 14A and B). In all the three cell lines, As₂O₃ alone induced much less modifications in hTERT expression, which yielded a telomerase activity above 50% of the control without any consequences on either telomere length or cell proliferation. In sharp contrast, the combination of the two compounds reduced hTERT mRNA and TA below the levels needed to prevent the reduction of telomere length and cell death.

Effect of ectopic hTERT-expression on the viability of retinoid maturation-resistant cells during ATRA/As₂O₃- treatments

Altogether, our results suggested that, after long-term treatment of maturation-resistant cells with ATRA plus As₂O₃, loss of telomerase activity and telomere shortening causes cell death. To substantiate this result, we investigated whether ectopic expression of hTERT could rescue NB4-LR1 and NB4-LR2 cells from cell death. NB4-LR1/hTERT-GFP and NB4-LR2/hTERT-GFP continued to proliferate without any change on cell viability, not only during long-term treatment with ATRA or As₂O₃, but also in case of co-treatments (Fig. 15)

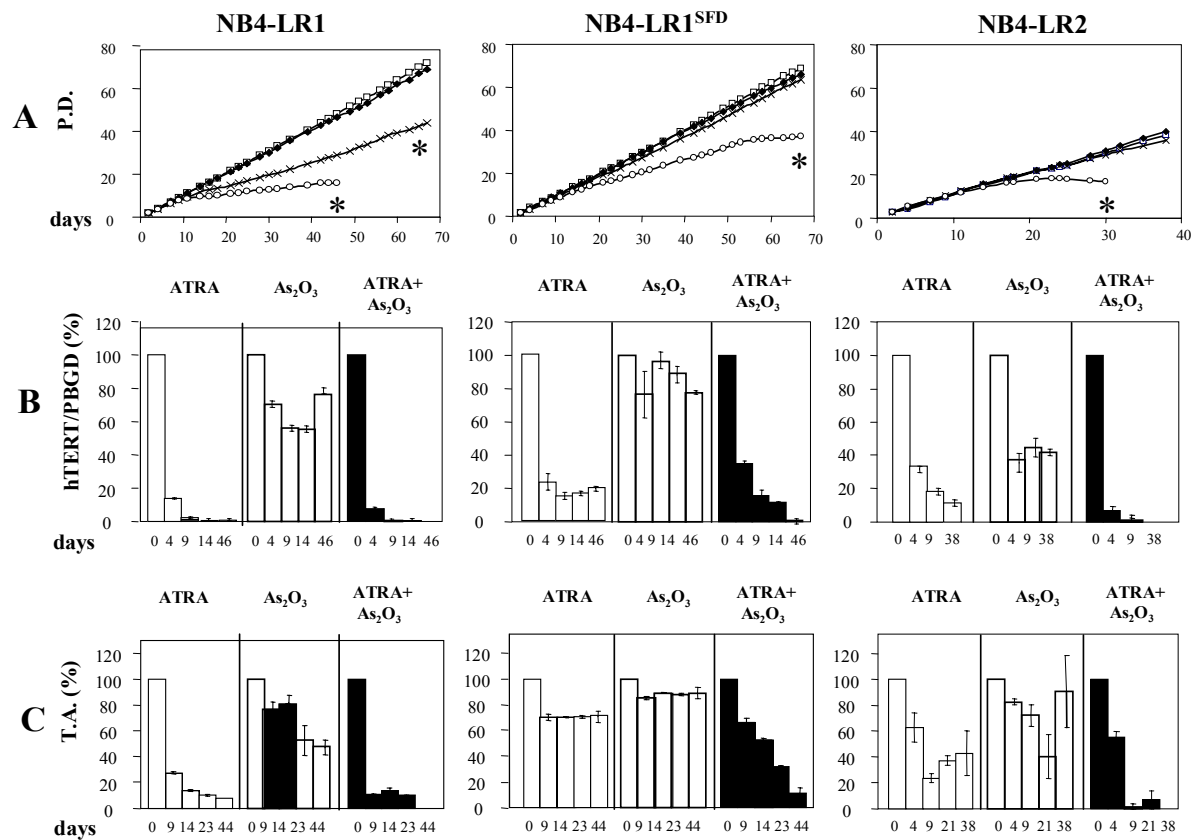


Figure 13:
ATRA and As_2O_3 synergize to induce growth arrest, hTERT downregulation, inhibition of telomerase activity and subsequent cell death.

A. Proliferation of cells cultured in the presence of medium alone (empty square), in the continuous presence of ATRA (1 μ M; cross), As_2O_3 (0.2 μ M; black diamond), or the combination of both drugs at these concentrations (empty circle). Cell proliferation was assessed as population doublings (PD). Asterisk indicates cultures that died after prolonged treatment with the indicated compounds.

B. At the indicated time, hTERT mRNA expression was quantified by fluorescence-real-time RT-PCR using LightCycler TeloTAGGG hTERT Kit. hTERT level was normalized to the expression of the housekeeping gene phosphobolinogen deaminase (PBGD).

C. Telomerase activity (TA) was measured with TRAPeze Elisa telomerase detection kit, and expressed as a percentage of that detected in untreated cells.

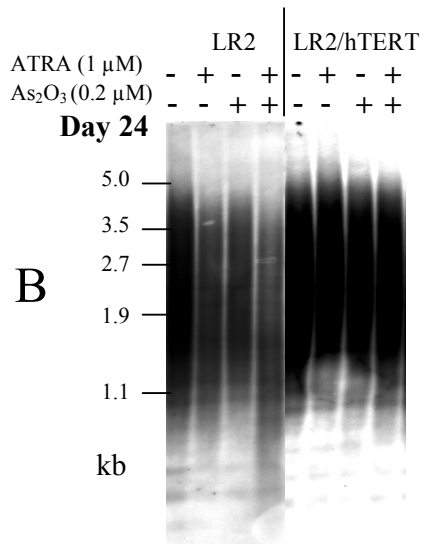
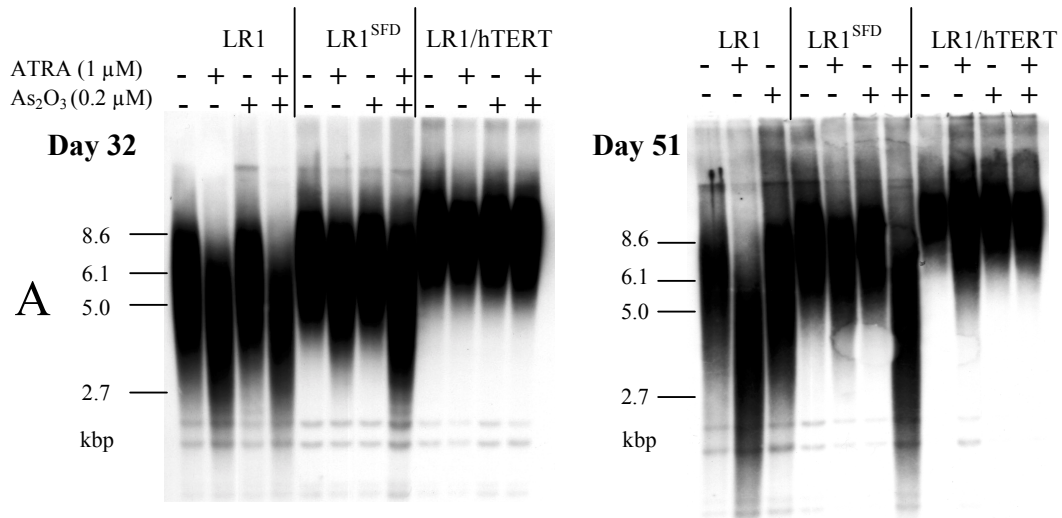


Figure 14:

*Average telomere length during long-term treatment of APL cell lines with ATRA/As₂O₃. Terminal restriction fragment lengths, measured using a non-radioactive chemiluminescent assay TeloTAGGG Telomere Length Assay (Roche Diagnostics) were determined after 32 days and 51 days (NB4-LR1 and NB4-LR1^{SFD} cells, **A**) and after 24 days of treatment (NB4-LR2 cells, **B**).*

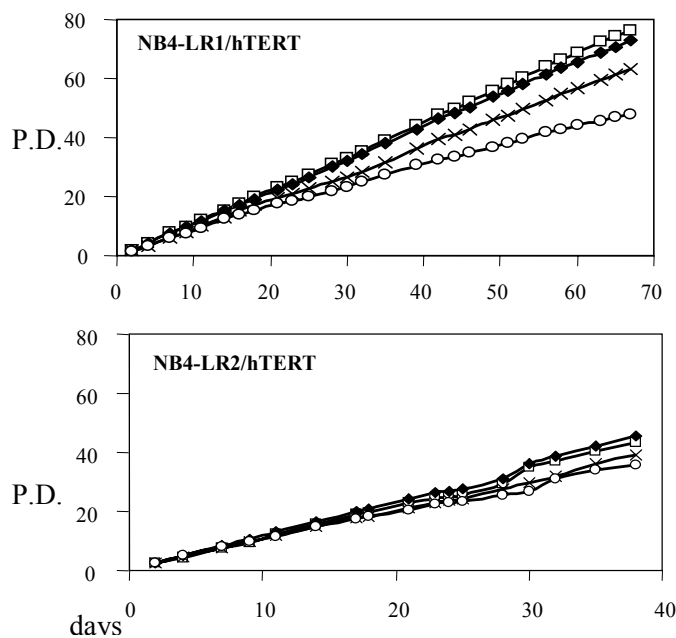


Figure 15:

Proliferation of ATRA and/or As₂O treated cells expressing ectopic hTERT. NB4-LR1/hTERT and NB4-LR2/hTERT cells were cultured in the presence of medium alone (empty square), the continuous presence of ATRA (1 μ M; cross), As₂O₃ (0.2 μ M; black diamond), or the combination of both drugs at these concentrations (empty circle). Cell proliferation was assessed as population doublings (PD).

Role of DNA-methylation in the ATRA-induced hTERT downregulation

Increased methylation of the promoter has been implicated in hTERT silencing during ATRA-induced differentiation in myeloid (147) and non-myeloid cell lines (148). Although literature of nature and significance of epigenetic changes caused by therapeutic arsenic is quite limited, several studies showed that methylation of appropriate genes is an important factor in arsenic carcinogenesis (149). Epigenetic silencing through DNA methylation could represent the point of convergence in telomerase downregulation observed after ATRA treatment. To test this hypothesis, we cultured NB4-LR1 cells in the presence of 250-500 nM of the methylation inhibiting agent 5'-azacytidine during 6 days. Concentrations higher than 500 nM caused massive cell death thus were not applicable. 1 μ M ATRA was added to the pretreated cultures, which were grown for additional 4 days, parallel to cells without ATRA. hTERT expression was determined with quantitative RT-PCR from total RNA of the harvested cells. Levels of telomerase catalytic subunit mRNA did not change upon AzaC treatment, but decreased significantly after receiving ATRA irrespective of pretreatment with the methylation inhibitor (Fig. 16).

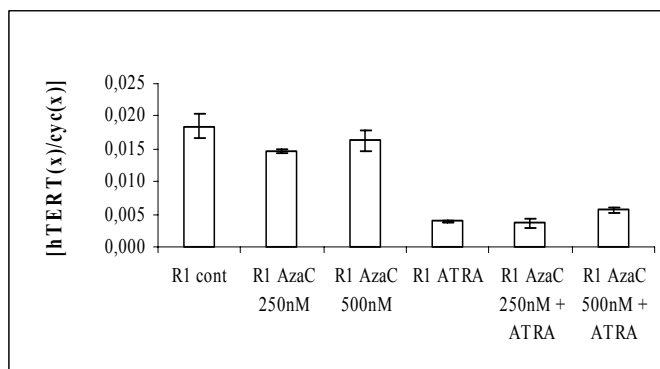


Figure 16: Effect of the DNA-methylation inhibitor (AzaC) on hTERT expression.

NB4-LR1 cells were grown in AzaC containing medium, then treated with 1 μ M ATRA. hTERT expression was analysed by real-time RT-PCR using a Taqman assay and normalized to the mRNA level of cyclophilin. AzaC pretreatment had no effect on ATRA-induced downregulation of hTERT.

Role of histone acetylation ATRA/As₂O₃-induced hTERT repression

Histone modification is another universal mechanism employed by eukaryotes for gene regulation, and plays a central role in retinoid-induced trans-activation. We tested therefore the changes in acetylation of histones 3 and 4 (H3 and H4) associated with the hTERT proximal promoter (-712/-435) with chromatin immunoprecipitation (ChIP). Samples were collected from day 2 and day 9 of ATRA (1 μ M)/ As₂O₃ (0.2 μ M) treatment. RAR β , being a direct RAR α target gene in NB4 cells (150), could be followed as positive control of the treatment. Parental cell line NB4 underwent differentiation upon ATRA treatment, which was accompanied by decreased H3 and H4 acetylation. Changes seen after 2 days were already present after 4h (Fig. 17).

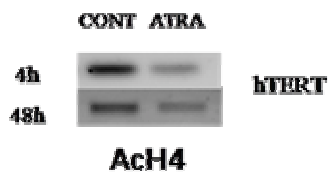


Figure 17 :Changes in histone acetylation during maturation of NB4 cells

NB4 cell were differentiated with 1 μ M ATRA, and histone acetylation of hTERT promoter was assayed with ChIP.

Acetylation of the hTERT associated histones decreased visibly as soon as 4h after adding ATRA.

In the maturation resistant NB4-LR1 cells, harvested at day 2, acetylation status was unchanged. At day 9, however, acetylation of both H3 and H4 decreased in ATRA-treated cells, and diminished even more in cultures receiving combinational treatment of ATRA and As₂O₃. As₂O₃ alone did not cause any changes (Fig 18A).

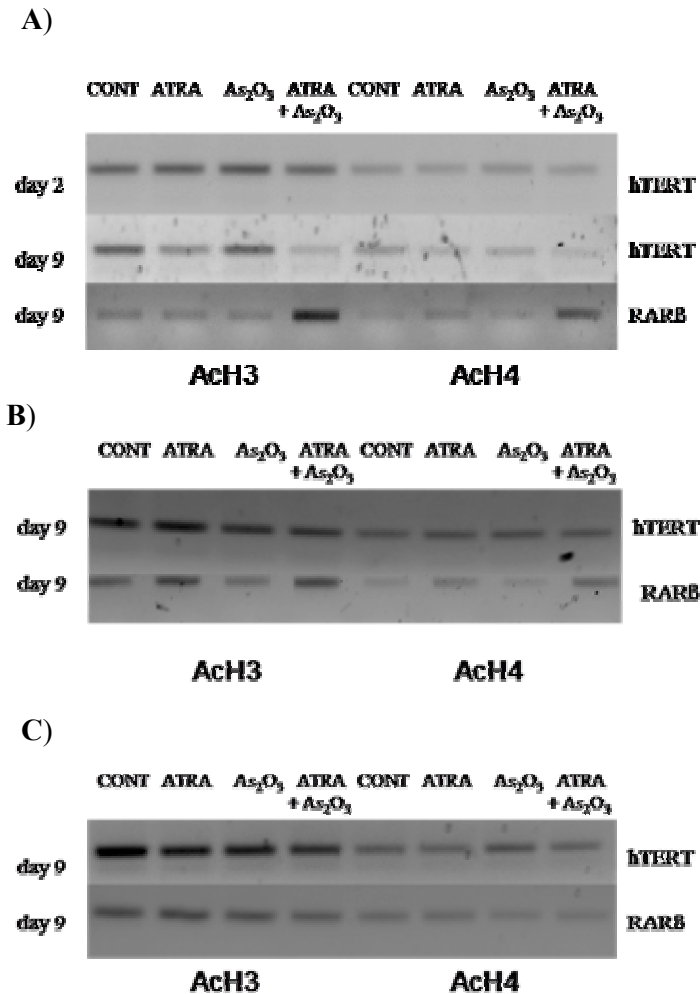


Figure 18.: Changes in histone acetylation during treatment with ATRA (1 μ M) and As₂O₃ (0.2 μ M)

ChIP assay was performed with cells collected at the indicated days using anti-AcH3 and anti Ach4 antibodies. PCR following immunoprecipitation amplified hTERT promoter or RAR β promoter respectively. Treated cell lines were A) NB4-LR1,

B) NB4-LR2

C) NB4-LR1^{SFD}

RAR β promoter used as control showed that decrease of acetylation is not a general phenomenon- liganded RAR α activated RAR β and caused histone acetylation, in good agreement with the literature. It is worth to note, that co-treatment was more effective, than ATRA alone, also in the transcriptional activation.

In treated NB4-LR2 populations (Fig. 18B) the histones in the control gene promoter presented similar changes to those seen in NB4-LR1, but hTERT associated H3 and H4 acetylation was not decreased. Histones on telomerase catalytic subunit promoter in NB4-R1^{SFD} (Fig. 18C) were also not altered after 9 days of combinational treatment. Interestingly, in these cells histones at the RAR β promoters were refractory to changes when treated with ATRA and/or arsenic.

As manifest changes in the acetylation status could not even be induced with those treatments, that were efficient to downregulate hTERT in NB4-R2 and NB4-R1^{SFD} cells, we asked whether differentiation is able to cause the same hypoacetylation as in NB4 cells. NB4-LR1^{SFD} is resistant to ATRA induced maturation, but combination of 1 μ M ATRA and 200 μ M CPT-cAMP (a membrane-permeable cAMP analogue) is able to differentiate these cells.

After 2 days of treatment, hTERT expression decreased as predictable, but decrease in acetylation of it's promoter could not be observed. RAR β AcH3 in these samples was elevated.

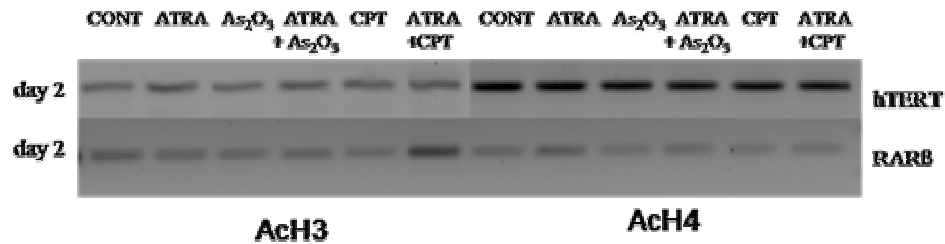


Figure 19.: Changes in histone acetylation during treatment of NB4-LR1^{SFD}
 Comparison of changes in histone acetylation after ATRA/As₂O₃ versus differentiation with ATRA/CPT [8-(4-chlorophenyl-thio)adenosine cyclic 3'5'-monophosphate]

DISCUSSION II.

Effect of ATRA and As₂O₃ on telomerase in maturation resistant APL cells

The mechanisms underlying the chemopreventive and chemotherapeutic properties of ATRA, as well as the improved efficacy of ATRA/As₂O₃ compared to single drug treatments among APL patients are still unknown. Our findings provide direct evidence that telomerase targeting can represent a likely new mechanism by which ATRA/As₂O₃ therapy can exert its action. We knew previously that telomerase-dependent cell death can be obtained in NB4-LR1 cell line, resistant to ATRA-induced maturation, by long-term treatment with ATRA (131). In NB4-LR2 similar effect was seen with the combination of specific ligands for RAR α and RXR (146). We demonstrated here, that only the combination is effective to induce a cellular death response in all the three maturation-resistant cell lines (NB4-LR1, NB4-LR2, NB4-LR1^{SFD}, Fig. 20) even though ATRA alone was able to regulate telomerase expression to some extent at a molecular level.

Although As₂O₃ alone in higher concentration has been shown to be enough to induce apoptosis (115), and literature data suggest that micromolar doses could decrease hTERT in a biological significant degree (151), our low-dose (0.2 μ M) application proved to be insufficient to influence long-term cell viability. It should be noted that modulation of TA and induction of cell death by ATRA/As₂O₃ in NB4-LR1 cells occurred faster than in cells treated with ATRA alone, although both treatments were able to eliminate the entire cell population.

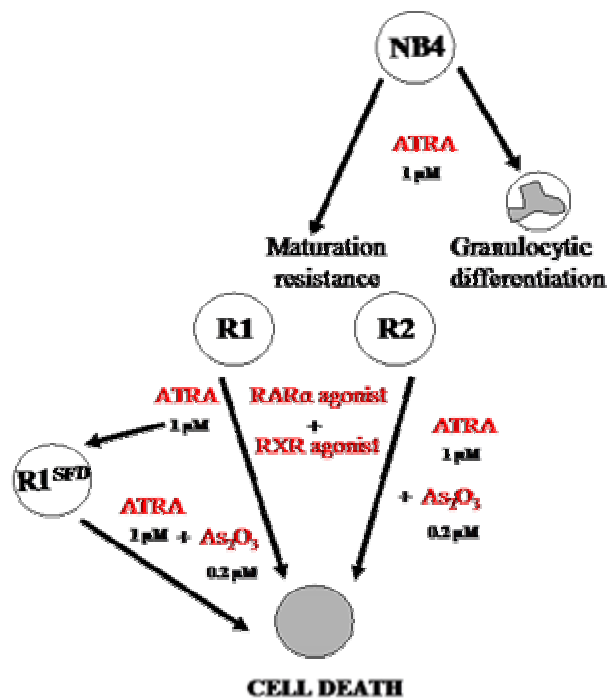


Figure 20.: Cellular response of acute promyelocytic leukemia cell lines to natural and synthetic retinoid ligands and their combinations.

The shorter period of time required to induce cell death in NB4-LR2 cells could be explained by the shorter telomere of these cells, compared to the two others (see TRF comparatively in Fig. 14 A and B). As a significant portion of the telomeres of the NB4-LR2 cells is expected to be at critical stage of shortening, even a small erosion (compared to what observed in NB4-LR1 and NB4-LR1^{SFD} using TRF assay) could be enough to trigger crisis and death of these cells. These results indicate that cell death induced by ATRA/As₂O₃ combination is mainly due to the decrease in TA, which is itself the consequence of hTERT downregulation. Importantly, we demonstrate that ectopic hTERT expression is sufficient to prevent cell death induced by ATRA/As₂O₃ combination, further supporting the contention that the therapeutic action of this combination of drugs relies on a telomerase and telomere dependent mechanism.

Interestingly, NB4-LR1 cells with ATRA alone proliferate at day 32 with telomeres as short as those of the cells receiving combination treatment and have already a decreased viability at this time point. It has been recently shown that not only the overall telomere length but also the length of the telomeric 3' single-strand overhang are important factors in triggering cell death. Since the TRF-method used in our experiments gives no information on the integrity of the 3'-overhang, it is not excluded that As_2O_3 in combination with ATRA induces some kind of telomere dysfunction. As a matter of fact, higher doses of arsenic have been shown to enhance the formation of tetra-stranded t-loop junction, which has been suggested to be a result of single-strand overhang loss (152). The telomerase-independent mechanism of the combination is also indicated by the reduced cell proliferation upon 40 days in NB4-LR1/hTERT-GFP cells (Fig. 15). Furthermore, we have to keep in mind that telomerase is endowed with survival functions independent of telomere maintenance, thus, the diminution in hTERT protein level, by eliminating it as a surviving factor, can also contribute to the effect observed in the parental cells without excessive amount of hTERT due to ectopic expression. However, the duration of the lag period before the induction of cell death suggest that telomere shortening is actually an important factor in the observed effect.

Converging mechanisms of ATRA/ As_2O_3 to influence hTERT transcription

Molecular network involved in the ATRA/Arsenic induced downregulation of hTERT may have several members. Approaches like high throughput genomic and proteomic assays, can provide a first list of interplayers. Systemic evaluation has already been conducted, but just focused on changes during differentiation of NB4 cells (153) and not on the effect of chronic treatment of maturation resistant cells.

Degradation of PML-RAR α

Mechanisms responsible for ATRA-induced telomerase downregulation in the absence of differentiation could not be deciphered yet. Although causal relation between degradation of PML-RAR α and changes in gene expression followed by ATRA/As₂O₃ administration cannot be doubted, it is clear that it can not solely account for the presented downregulation of hTERT, since the breakdown of the chimeric protein following ATRA treatment alone is already rapid and occurs to the same extent in NB4-LR1 and NB4-LR1^{SFD} cells, despite of different biological response (cell death or survival, respectively (130).

Transrepression

Classical consensus sequence for the heterodimeric RAR α /RXR receptor (5'-AGGTCA-3') is not present in the hTERT promoter, but in light of the recent findings describing a new cis-acting element for retinoids found in the promoter of the Burkitt's lymphoma receptor 1 gene of the myeloid cell line HL-60 (154), we have to realize that other binding sequence motif with novel binding complexes may exist. Little is known anyhow about ligand induced gene-repression at the molecular level. A nice example of transrepression by nuclear receptors has been demonstrated for vitamin D dependent 1 α -hydroxylase repression (155). In that case a sequence specific activator was able to bind liganded VDR/RXR, which caused the switch from co-activators to HDAC co-repressor complex. A similar molecular structure could also be imaginable for ATRA-dependent hTERT downregulation.

Anti-AP1 activity

Retinoids modulate gene expression by two different ways. First by the binding of RAR/RXR heterodimers to response elements, followed by transactivation. Secondly, as transrepressive factors that negatively affect proliferation-associated genes via their

ability to functionally interact with transcriptional factor AP1 (156). The AP1 transcription complex consists of homodimers and heterodimers of members from the fos and jun families and activates target genes by binding with high affinity to particular DNA cis-element. The regulation of AP1 activity is complex and occurs at different levels. First, through differential expression of individual members from jun and fos families, which will determine the composition of AP1 dimer (157) Second, AP1 activity is regulated at posttranslational levels by several external stimuli mainly involving mitogen-activated protein kinase (MAPK) cascades. Mechanisms by which retinoids exert anti-AP1 activity is still unclear. Proposed mechanisms included inhibition of jun N-terminal kinase or triggering the assembly of distinct AP1 complexes, different from those in unstimulated cells (158), and sequestration of c-jun/c-fos sequestration through physical interactions with RAR (159). Although anti-AP1 effect in telomerase downregulation can not be excluded and would be an interesting question to be investigated, there some main concerns that make it improbable. One of them is the report about NB4 cells treated with anti-AP1 retinoids (GALDERMA CD2409), which had just a modest effect on cell growth and differentiation. It should be noted though, that treatments were just conducted on short term (160). Next contradictory fact is the requirement of liganded RXR to repress hTERT (146), since anti AP1 effects do not require the heterodimeric nuclear receptor and RAR is enough to produce full effect (161). Interestingly, most recent findings suggest that in an unusual manner, AP1, a traditionally positive regulator of proliferation has a repressional function on hTERT (162).

Epigenetic modifications

Treatment of several telomerase positive cell types with methylation-inhibiting agents led to a decrease of the catalytic subunit, while others reported an increased

expression. In NB4-LR1 we did not observe any kind of effect. Retinoids are known to decrease methylation by inhibiting the expression of methyltransferases (163). Realizing, that ATRA hardly acts directly, other transcription factors, affected by retinoid signaling, could be as well involved in changes modifying the chromatin. Inhibition of DNA-methylation by pretreating NB4-LR1 with AzaC, proved neither preventive from ATRA-induced transcriptional repression, nor enhanced the effect of it. Role of DNA-methylation in hTERT biology seems to differ from cell to cell. In our cell lines no evidence was found for its role in telomerase regulation.

Acetylation of promoter proximal histones is associated with gene expression, while transcriptional silencing, seen for hTERT in normal human somatic and ALT cells, can be induced by transcription factors that recruit HDAC. Early downregulation of hTERT during differentiation is a well known phenomenon, and has also been shown to be associated with histone deacetylation previously (30). Parental NB4 cells treated with ATRA during two days, demonstrated the same pattern. In NB4-R1 cells ATRA and arsenic act cooperatively in increasing histone acetylation of RAR β promoter and the presence of the same synergism can be observed when the two agents decrease acetylation associated with hTERT. It is worth to keep in mind that the first is a direct RAR α target gene while the latter is supposed to be effected indirectly. PML-RAR α of NB4-LR2 harbors a mutation, generating an in-phase stop codon, thus truncated form of PML-RAR α which has lost its C-terminal end. Fusion mutant exerts a dominant negative effect on wild-type PML, PML-RAR α and RAR α transcription activity (164). Its likely that the altered fusion protein is less effective in the formation of activating nucleoprotein complexes and as a consequence improper HDAC activity can be observed after ATRA and combinational treatment. NB4-LR1^{SFD} is quite unique, as hyperacetylation of RAR β promoter was just present in cell undergoing differentiation.

There might be several explanations. Taking the fact that genetic background of the altered phenotype is not found yet, we can just guess, that the reason of the observed deviations might be a defect in the retinoid signaling pathway, not only affecting hTERT. Preexisting histone modifications, like preacetylation, were shown to interfere with chromatin methylation (165). Since we had not the opportunity yet for the quantitative comparison of the cell lines, we can just guess, that preexisting epigenetic changes could account for retinoid resistance.

Although we could not show clear correlation between histone acetylation state and biological response to the treatment, it is important to keep in mind that epigenetic regulation functions through a “histone-code”, thus a combination of different non-independent histone modifications, what highlights the future importance of working with a palette of modifications. It is as well undoubted that using non quantitative methods, like our experimental layout, makes us impossible to speak about kinetics and amplitude of changes, we are just able to see a kind of endpoint, and make statements about manifest changes.

Taking together, we are unable to give a clear explanation how ATRA regulates hTERT, and how the converging pathways with As_2O_3 are able to cooperate. The main concern about making any suggestion is the time course of our observation: even in case of a secondary effect, a retarded response like seen in NB4-LR1^{SFD} can not be explained with our today's knowledge.

CONCLUDING REMARKS

Our observations made with ATRA/As₂O₃ and chimeric oligonucleotides highlight a few important points for telomerase as future cancer drug target.

Although there are already studies focusing at minimum levels of telomerase activity for tumorigenesis (166), the notion of thresholds necessary for telomere shortening and cell death are stated rarely. *The first threshold* features to which extent hTERT mRNA expression has to be decreased to obtain an efficient reduction of TA. *The second one* identifies the level of enzyme activity for which telomerase inhibition resulted in a sufficient shortening of telomeres to cause cell death. The biological significance of these two distinct thresholds has to be taken into account for the development of telomerase inhibitors targeting either hTERT mRNA expression or telomerase activity itself. In fact, focusing solely on a relative inhibition of telomerase may overestimate the therapeutic efficacy of telomerase inhibitors. Indeed, tumor cells can be responsive to a drug, in terms of downregulation of hTERT and decrease in TA, without demonstrating any modification in proliferation and viability. Importantly, it is also clear that these thresholds, as well as the outcome of telomerase inhibition, may vary depending on the physiology of the targeted cancer cells.

A number of oligonucleotides were tested as telomerase inhibitors mostly interacting with the template region of hTR. The (s⁴dU)_nAS type inhibitors, developed by us, have a quite unique feature; they interact with the protein subunit of the telomerase enzyme, too. Although, such telomerase inhibitors were also developed by others (123), our family of inhibitors is the only one, which is able to form covalent linkage with proteins. The further development of (s⁴dU)_nAS type type-inhibitors is possible because the antisense stretch offers an excellent target for further modifications increasing the stability of the inhibitor against nucleases as well as enhancing the

stability of the double helix formed with hTR. Molecules, tightly bound to telomerase could also be used to map three dimensional organizations of functional domains, since structure of the enzyme is not fully evaluated yet.

Considering the therapeutic utility of oligonucleotide-type telomerase inhibitors, including ours, the low toxicity of these agents allows a treatment may be applied for a long period of time. Considering problems of stability and penetration systemic application might not be appropriate for oligonucleotides, but local administration as a part of the post-surgical treatment of should be considered for certain malignancies.

Our findings provide evidence that telomerase targeting can represent a likely new mechanism by which ATRA/As₂O₃ therapy can exert its action in APL patients. The synergism between these two agents highlights important aspects of their possible integration into the future clinical protocols (118). Although benefit of arsenic trioxide was shown first in APL patients in the first or subsequent relapse after ATRA and chemotherapy, a recent study showed (116) the clear benefit of arsenic-ATRA combination in newly diagnosed APL in induction and maintenance therapy. We suggest that the success of the latter trial can be also explained by the fact, that the administration of the combination was sustained over the period required for complete remission, which is around 30-40 days in general. In light of our results, presented here, the time span of induction therapy seems to be not always enough to obtain critical telomere shortening, thus resistant clones could escape, while sustaining the therapy with the combination would also allow the eradication of multiple kinds of resistant cells. It seems, that long-term low-dose combinational treatments could lead to a favorable outcome in APL patients, with the enhanced benefit of minimal toxicity, as As₂O₃ concentration used in our study (0.2 μM) is much lower than the therapeutic serum levels of arsenic during standard and alternative therapeutic protocols, which are

settled to yield a concentration around 1 μM , with peak concentrations of 3-6 μM (167). As arsenic trioxide as postremission therapy seems to be favorable in patients receiving this drug after relapse, our experiments indicate, that it might be used as well in low dose in combination with ATRA. The fact, that deregulation of hTERT expression is a mechanism that is not restricted to APL, clearly indicates, that the eradication of the residual cells will likely require pharmacological treatment targeting telomerase.

Combinational treatments raise the idea of a more general concept to investigate other combinations, in order to know, whether their extended administration, influencing telomerase in a convergent manner, could not be of therapeutic benefit. This type of strategies might involve combinations of drugs targeting telomere accessibility/function and hTR/hTERT component of the telomerase holoenzyme (71, 81, 100), co-treatments against hTR and hTERT (78), combinations that influence regulation of hTERT on different levels, as well as the usage of molecules that exert synergy by targeting the same regulation event by different mechanisms Especially attractive would be the applications that include clinically approved drugs, which have not been used before in extended protocols, but have already the permission to be used for human therapy. Since malignant cells with elevated telomerase activity can be resistant to anti-cancer agents, and this resistance seems to be reversed by concomitant treatment with telomerase inhibitors (168) several types of drug resistant cancers could represent a rational target.

Although the complexity of telomerase regulation and malignant transformation, as well as the individuality in the clinical response show clearly that treatment protocols, similar to our experimental layouts may not be effective in all cases, and not applicable as first and only treatment, our data encourage their integration into extended trials performed *in vitro* and in the clinic.

SUMMARY

Immortalization is an indispensable step in oncogenesis. Most of the human malignant cells attain their immortality through stabilization of their telomere length by reactivating telomerase enzyme, a specialized reverse transcriptase. I presented in my thesis two promising approaches to target telomerase in immortal human cell lines using combinational strategies.

I. TELOMERASE DEPENDENT CELL DEATH USING CHIMERIC OLIGONUCLEOTIDES AS TELOMERASE INHIBITORS

1. We designed an oligonucleotide family, composed of a 13mer antisense sequence against the hTR template part and a 3' or 5' (s⁴dU)_n moiety, possibly interacting with the protein subunit of human telomerase and we characterized the oligonucleotides by meaning of their inhibition parameters.
2. We proved, that the derivate (s⁴dU)₈AS_(PS), shown to be specific and effective as well, can enter human cells.
3. Long-term treatment with (s⁴dU)₈AS_(PS) caused the death of the entire treated immortal cell population.

II. TELOMERASE DEPENDENT CELL DEATH USING ATRA AND As₂O₃

1. We proved that all-trans-retinoic acid and As₂O₃ is able to downregulate hTERT expression even retinoid resistant cell lines. The decrease in expression diminished telomerase activity and caused telomeres to shorten.
2. Long term low dose combinational treatment was sufficient to cause cell death.
3. Synergistic effect of all-trans-retinoic acid/As₂O₃ treatment could not be exclusively explained by the modification of the chromatin structure.

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Chimeric oligonucleotides are patented by Aradi, Tarkanyi, Horvath, Szatmari, Segal-Bendirdjian: [„Oligonucleotide chimeras as reverse transcriptase or telomerase inhibitors.”, Hungarian patent application No.: P05 00154]

ABBREVIATIONS

ALT	- Alternative Lengthening of Telomeres
APL	-Acute Promyelocytic Leukemia
ATRA	-All-Trans Retinoic Acid
AzaC	-5'-azacytidine
ChIP	-Chromatin ImmunoPrecipitation
CR	-Complete Remission
DNP	-Dinitrophenyl
FCS	-Fetal Calf Serum
MTS	-Modified Telomerase Substrate
PBS	-Phosphate Buffered Salina
PCR	-Polymerase Chain Reaction
PKC	-Protein Kinase C
PML	-Promyelocytic Leukemia Protein
RARα	- Retinoic Acid Receptor alpha
RXR	-Retinoid X Receptor
TRAP	-Telomeric Repeat Amplification Protocol
TP-TRAP	-Two-Primer Telomeric Repeat Amplification Protocol
TRF	-Terminal Restriction Fragment

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