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

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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

Telomeres are terminal protein-DNA complexes that stabilize chromosomal ends preventing them from aberrant recombination and being recognized as DNA double-strand breaks. 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. 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-loop (displacement-loop). This structure, stabilized by telomeric proteins, seems to exist to protect against nuclease degradation and recombination.

“END-REPLICATION PROBLEM” OF THE LINEAR CHROMOSOMES

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.
TELOMERE MAINTAINING MECHANISMS

Telomerase

Telomerase is a ribonucleoprotein complex that includes a 451 base long RNA (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. Telomerase has been shown to add repeats to the telomeres in a processive manner: 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: the 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. 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. 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.
TELOMERE HYPOTHESIS OF CANCER

Classical role of telomerase is to maintain telomeres, by copying the template sequence of its RNA moiety. 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. 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 is activated in more than 85% of malignant tumors. 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. Immortal cells prefer telomerase over alternative mechanisms. This indirect evidences suggested 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.
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. 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. Continuous telomere maintenance is predominantly due to the transcriptional upregulation of hTERT expression, which is governed by complicated regulatory pathways. The chromatin context as well plays an important role in the gene expression, and emerging evidence suggests its implication in hTERT regulation. Besides gene expression, growing knowledge of telomerase biology identified several processes where the amount of catalitically active protein was regulated by splicing. Deletion variants that remove regions of the conserved reverse transcriptase domain of hTERT encode a catalitically inactive protein. As in case of many enzymes, postranslational modifications are used to control hTERT activity in certain pathological and physiological processes.
TELOMERASE IN CLINICS

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. 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.

TELOMERASE INHIBITORS

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. hTR was a quite obvious target for telomerase inhibition strategies, as its function as template makes it accessible also for inhibitory strategies. 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. Classical antisenses, molecules directed against hTERT mRNA or pre-mRNA, are less established.

Pharmacological resetting 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/ vitaminD$_3$), thus hTERT downregulation. Inhibitors of Protein kinase C or chaperone Hsp90 were also tested to inhibit correct posttranslational processing of the telomerase holoenzyme.
ACUTE PROMYELOCYTIC LEUKEMIA

Acute promyelocytic leukemia (APL), a subtype of acute myeloid leukemia is characterized by the accumulation of abnormal promyelocytes, disseminated intravascular coagulation and the chromosomal translocation t(15;17) which fuses retinoid receptor-alpha (RARα, on chromosome 17) to the promyelocytic leukemia gene (PML) on chromosome 15 and leads to the expression of the PML-RARα fusion protein. This aberrant transcription factor binds to the target genes in the form of oligomers or in complexes with RXR, recruits histone deacetylases (HDAC) and DNA-methyltransferases, leading to deregulation of retinoic acid target genes, that play critical roles in myeloid differentiation. As a result maturation is blocked in the promyelocytic stage. Physiological doses of retinoids are insufficient to trigger the release of the corepressor-HDAC complex, but pharmacological doses (10^-6 M) can induce the coactivator-corepressor change. 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.

First studies showed efficacy of As₂O₃ as single-agent therapy in relapsed or refractory APL after ATRA and anthracycline treatment. ATRA and arsenic showed synergistic effect in vitro and no cross resistance in clinics, and furthermore, combinational induction therapy resulted in higher rate of molecular remission without increasing toxicity. Despite of encouraging trials, the success of the ATRA plus chemotherapy based induction pushes other protocols into the second line. Only patients with comorbid conditions are induced with ATRA/As₂O₃ combination. The palette of mechanisms underlying the cooperative action of ATRA and As₂O₃ on molecular level contains several members. ATRA as well as arsenic was shown to induce cleavage of the PML-RARα fusion protein and changes in gene expression.
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.

We intended to design, synthesize and study the \textit{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 planned to synthesize antisense (13mer) oligonucleotides carrying $(s^4dU)_n$ ($n = 4, 8, 16, 24$) at its 3’ or 5’ ends, 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 As$_2$O$_3$ 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/As$_2$O$_3$ 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/As$_2$O$_3$ to the modulation and/or degradation of PML-RAR$\alpha$ oncprotein 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 HL-60 cells. Main steps of the purification were: precipitation with 40% ammonium sulphate and anion exchange chromatography on DEAE–Sepharose column (Pharmacia). After chromatography, active fractions were pooled, dialyzed and re-purified on DEAE-Sepharose column.

Oligonucleotides

Chemically modified oligonucleotides used for inhibition studies, Cy5-labeled molecules and primers used in TP-TRAP assay were synthesized by standard phosphoramidite chemistry on a Gene Assembler Plus (Pharmacia) oligonucleotide synthesizer, and then purified by anion exchange chromatography. 3’-blocked (s^4dU)_8AS was synthesized using 3’-Spacer C3 CPG (Glen Research).

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. Experiments with protein extracts of the oligomer and ATRA/As_2O_3 treated cell cultures were performed with TRAPeze Elisa telomerase detection kit (Qbiogene) according to the manufacturer’s instruction.
**Oligonucleotide uptake studies**

A431 and 293T cells were transfected with Oligofectamine Reagent and Cy5-labeled oligonucleotides according to the manufacturer’s directions. Following a fixation/permeabilisation step, contours of the plasma membrane and the nucleus were visualized with Alexa Fluor 488-labeled anti-EGFR antibodies and ethidium bromide. Cellular localization of labeled inhibitors was determined by confocal laser scanning microscopy.

**Long-term treatment of cell cultures with chimeric oligonucleotides**

293T cells were treated with 1-3 µM \( (s^4\text{dU})_8\text{AS}_{(2PS)} \) in the presence of Oligofectamine. Cultures were counted, replated and transfected every second or third day. Cell proliferation was expressed as population doubling. Telomerase activity during treatment was monitored from samples subjected to TRAP-assay.

**Detection of senescence**

Senescent phenotype of treated cells was determined by cytochemical staining for senescence-associated-galactosidase.

**Acute promyelocytic leukemia cell-lines**

The maturation-resistant human promyelocytic leukemia cell lines NB4-LR1 and NB4-LR2, the selected subline NB4-LR1\( ^{\text{SFD}} \) (Saved From Death), the retrovirally infected NB4-LR1/hTERT-GFP and the NB4-LR2/hTERT-GFP sublines were treated with the respective drugs or their combination to yield the appropriate end-concentration: 1µM for ATRA and 0.2 µM for As\(_2\)O\(_3\). Every second or third day, cells were counted (Coulter Counter), and reseeded in cell culture medium containing the respective pharmacons. Effect of the treatments was monitored as following:

1) Cell proliferation was determined by serial cell counting with Coulter counter.
2) Real-time quantitative PCR (LightCycler technology and LightCycler teloTTAGGG hTERT Kit from Roche Diagnostics) for measuring the expression of telomerase catalytic subunit.

3) TRAP (Telomeric Repeat Amplification Protocol)-assay for quantification of telomerase activity (TRAPeze Elisa Telomerase Detection Kit, Qbiogen)

4) We measured the average telomere length as a telomerase-specific cellular response, with the help of the TRF (Terminal Restriction Fragment)-analysis (TeloTTAGGG Telomere Length Assay, Roche Diagnostics).

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

NB4-LR1 cells were treated with 5-azacytidine (AzaC). On day 6 the cultures were divided, and half of the cells were treated for 4 days with 1 uM ATRA in addition, while the other half was cultured just as before. hTERT expression was determined with quantitative PCR, performed using real-time PCR (ABI PRISM 7900, Applied Biosystems) and Taqman assays.

**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 on APL cells receiving ATRA (1 µM), As2O3 (0.2 µM), 8-(4-chlorophenyl-thio)adenosine cyclic 3’5’-monophosphate (200 µM) treatment in different combinations. Products from the ChIP assay were amplified using the primers flanking the region -712/-435 of the hTERT promoter. As a positive control a fragment of the RARß gene promoter was amplified. PCR products were analyzed on 2% agarose gels stained with ethidium bromide.
RESULTS and DISCUSSION I.

TELOMERASE DEPENDENT CELL DEATH USING CHIMERIC OLIGONUCLEOTIDES AS TELOMERASE INHIBITORS

1) Effect of 3' or 5' attached (s4dU)n on the telomerase inhibiting potential of a 13mer antisense oligonucleotide

The inhibitory features of (s4dU)35 prompted us to develop chimera oligonucleotides, presumably specific inhibitors of telomerase, composed of an antisense sequence (AS) and a 3' or 5' (s4dU)n (n = 4, 8, 16, 24) moiety, which is thought to interact with the protein subunit of human telomerase. First we determined the IC50 values for the inhibitors on partially purified telomerase using the TwoPrimer-TRAP assay. Control experiments for chimeric molecules included oligonucleotides comprising just the chemically modified component, scrambled molecules with 5' (s4dU)n's to prove sequence specificity and parent compounds for chemically modified chimeras to evaluate the role of the thiolated moiety (CnAS). Sequence specificity of AS-mediated inhibition was proven by scrambled control. We chose 10 min preincubation for the inhibitors with telomerase, because this period was measured to be obligatory and sufficient for the chimeras to exhibit maximal inhibitory potential.

Antisense oligonucleotides with 5' (s4dU)n's were more potent inhibitors either than the antisense alone or the 3’ modified ones. This seems to support the dual targeting hypothesis, namely that AS sequence of (s4dU)n AS inhibitors forms double helix with the template region of hTR, while (s4dU)n interacts with the protein part, and the two interactions enhance each other. 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. Increasing length of the thiolated moiety increased the telomerase inhibitory potential. The similar inhibitory activity of (s^4dU)_{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 high inhibitory potential of the (s^4dU)_{24}SCR which is not able to form base pairs with the hTR template sequence.

Specificity of the oligonucleotides was proven on HIV reverse transcriptase, which shares common motifs with hTERT. Chimeras with long modified moieties turned out not to be specific for telomerase. In contrast, AS and AS with 4 or 8 (s^4dU)s were weak inhibitors of the viral reverse transcriptase.

Considering is the physico-chemical nature of the interaction of thiolated oligonucleotides with the protein, there are two likely modes: (a) hydrophobic interaction, or (b) disulfide bond formation between properly localized cysteiny1 side chains on hTERT surface and reactive –SH, formed on the (s^4dU) base due to tautomeric conversion.

2) Cellular uptake of oligonucleotide inhibitors

The efficiency of antisense strategies is highly dependent on the cellular delivery of oligonucleotide agents. We examined therefore the cellular uptake of the inhibitors with confocal laser scanning microscopy using Cy5-labeled oligonucleotides. Chosen candidates for uptake studies were (s^4dU)_{4}AS and (s^4dU)_{8}AS, since their inhibition characteristics seem to be the most attractive for anti-telomerase strategies in cellular systems. Pictures obtained with the confocal microscope clearly showed the internalization of the chemically modified oligonucleotides. Labeled inhibitors were found in the cytosol and in the nucleus as well, with a characteristic perinuclear enhancement.
3) Sustained \((s^4dU)_8 AS_{(2PS)}\) treatment of telomerase positive cells

We treated a short-telomere variant of 293T cells with 1-3 µM \((s^4dU)_8 AS_{(2PS)}\), and Oligofectamine Phosphorothioate modification has been introduced to enhance inhibitor stability in the cellular environment, without increasing nonspecific association known to be a property of fully phosphorothioate oligonucleotides. Chimeric oligomeres were able to decrease telomerase activity in a dose dependent manner which was sufficient to cause the death of the treated cell population after continuous long-term administration. \((s^4dU)_8 SCR_{(2PS)}\) in similar conditions had just a slight effect. The delayed onset of the phenotypic effect during oligonucleotide treatment can be explained by the fact that these inhibitors suppose to decrease the activity of 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 ß-galactosidase activity.

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. 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. 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 and DISCUSSION II.

TELOMERASE DEPENDENT CELL DEATH USING ATRA AND As$_2$O$_3$

1) Effect of ATRA/As$_2$O$_3$ on the telomerase expression of the maturation resistant promyelocytic leukemia cell lines

Majority of therapy-naive acute promyelocytic leukemia (APL) cells as well as NB4 APL cell line enters granulocytic differentiation upon ATRA treatment. 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. Repression of telomerase with subsequent cell death can also be obtained using synthetic RAR$\alpha$ and RXR selective agonists not only in NB4-LR1 but also in “ATRA-unresponsive” NB4-LR2 cells, harbouring a dominant negative, truncated PML/RAR$\alpha$. Subline NB4-LR1$^{SFD(Saved From Death)}$ retains a functional pathway of telomerase downregulation associated with maturation, but its hTERT expression fails to respond to ATRA or selective agonists. 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 As$_2$O$_3$ inhibit hTERT expression, we wondered whether this combination, proven to be effective in clinic, could synergistically affect the telomerase homeostasis of ATRA-resistant cells. One critical factor that limits the utility of As$_2$O$_3$, 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 As$_2$O$_3$ at low concentration (0.2 $\mu$M) in the presence or absence of ATRA (1 $\mu$M). Our observations showed that long-term exposition to the combination of ATRA and As$_2$O$_3$ resulted in telomerase catalytic subunit downregulation, leading to a marked decrease in
telomerase activity, and finally to telomere shortening in all the maturation resistant APL cell lines included in our experiment.

2) Viability of maturation resistant APL cells after long-term ATRA/As$_2$O$_3$ administration

ATRA alone was able to regulate telomerase expression to some extent at a molecular level, but similar to the previous results, it was not sufficient to eliminate ATRA-resistant NB4-LR2 and NB4-LR1$_{SFD}$ cell lines. We demonstrated, 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$_{SFD}$). 0.2 µM As$_2$O$_3$ alone had no effect either on proliferation or viability in none of the cell populations.

To substantiate the result, that observed phenotypic effect is due to telomerase downregulation, 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, thus further supporting the contention that the therapeutic action of ATRA/As$_2$O$_3$ combination relies on a telomerase and telomere dependent mechanism. 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.

Our findings provide direct evidence that telomerase targeting can represent a likely new mechanism by which ATRA/As$_2$O$_3$ therapy can exert its action in APL patients. In light of our results 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 during the maintenance phase would also allow the eradication of multiple kinds of resistant cells that would emerged. We suggest that the success of recent
clinical trials with arsenic-ATRA combination in newly diagnosed APL can be also explained by the fact, that in contrast to the previous protocols, the administration of the combination is sustained over the period required for complete remission, which is around 30-40 days in general.

3) Role of epigenetic modifications in the ATRA-induced hTERT downregulation

Mechanisms responsible for ATRA-induced telomerase downregulation in the absence of differentiation could not be deciphered yet. Although causal relation between degradation of PML-RARα and changes in gene expression followed by ATRA/As₂O₃ administration cannot be doubted, it is clear that it does not solely account for the presented downregulation of hTERT. The breakdown of the chimeric protein following ATRA treatment alone, is already rapid and occurs to the same extent in NB4-LR1 and NB4-LR1SFD cells, despite of different biological response (cell death or survival, respectively). 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. Classical consensus sequence for the heterodimericRARα/RXR receptor is not present in the hTERT promoter, but other binding sequence motifs with novel complexes may exist.

Increased methylation of the promoter has been implicated in hTERT silencing during ATRA-induced differentiation in myeloid and non-myeloid cell lines. Epigenetic silencing trough DNA methylation could represent the point of convergence in telomerase downregulation observed after combinational treatment. To test this hypothesis, we cultured NB4-LR1 cells in the presence of the methylation inhibitor, 5’-azacytidine (AzaC) during 6 days. 1 μM ATRA was added to the pretreated cultures, which were grown for additional 4 days,
parallel to cells without ATRA. 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.

Histone modification is another universal mechanism employed by eukaryotes for gene regulation, and plays a central role in retinoid-induced transactivation. We tested therefore the changes in acetylation of histones 3 and 4 (H3 and H4) on the hTERT proximal promoter (-712/-435) with chromatin immunoprecipitation (ChIP). Samples were collected from day 2 and day 9 of ATRA (1 µM)/ As$_2$O$_3$ (0.2 µM) treatment. RARβ, being a direct RARα target gene in NB4 cells, 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.

In the maturation resistant NB4-LR1 cells, harvested at day 9, acetylation of both H3 and H4 at hTERT promoter decreased in ATRA-treated cells, and diminished even more in cultures receiving combinational treatment of ATRA and As$_2$O$_3$. Liganded RARα activated RARβ and caused histone acetylation, in good agreement with the literature. In treated NB4-LR2 populations hTERT associated H3 and H4 acetylation was not decreased. PML-RARα of NB4-LR2 harbors a mutation, generating an in-phase stop codon, thus truncated form of PML-RARα. Fusion mutant exerts a dominant negative effect on wild-type PML, PML-RARα and RARα transcription activity. 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. Histones on telomerase catalytic subunit promoter in NB4-R1$_{SFD}$ were also not altered after 9 days of combinational treatment. Interestingly, in these cells histones at the RARβ promoters were also refractory to changes when treated with ATRA and/or arsenic. During differentiation of NB4-R1$_{SFD}$ cells, induced
by the combination of 1 µM ATRA and 200 µM cAMP analogue CPT-cAMP, hTERT expression decreased as predictable, but decrease in acetylation of it’s promoter could not be observed. RARβ AcH3 in these samples was elevated. NB4-LR1<sup>SFD</sup> is quite unique, as hyperacetylation of RARβ promoter was just present in cells 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. One of the explanations is, that preexisting epigenetic changes could account for retinoid resistance. 

Taking together, clear correlation between histone acetylation state and biological response to the treatment could not be shown, thus we are still unable to give a clear explanation how ATRA regulates hTERT, and how the converging pathways with As<sub>2</sub>O<sub>3</sub> 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<sup>SFD</sup> cannot be explained with our today’s knowledge.
CONCLUDING REMARKS

Our observations made with ATRA/As$_2$O$_3$ 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, 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 telomerase activity. The second one identifies the level of enzyme activity for which telomerase inhibition resulted in a sufficient shortening of telomeres to cause cell death. Focusing solely on a relative inhibition of telomerase may overestimate the therapeutic efficacy of telomerase inhibitors. It is also clear that these thresholds, as well as the outcome of telomerase inhibition, may vary depending on the targeted cancer cells.

The fact, that deregulation of hTERT expression is a mechanism that is a quite common in malignant proliferative diseases, clearly indicates, that complete eradication of the cancer cells will likely require beside the classical therapeutic approaches pharmacological treatment targeting telomerase activity as well. 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' \((s^4dU)_n\) moiety, possibly interacting with the protein subunit of human telomerase, and characterized the in vitro inhibition parameters of the members.

2. We proved, that potent and specific derivate \((s^4dU)_8AS(PS)\) can enter human cells.

3. Long-term treatment with \((s^4dU)_8AS(PS)\) 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 as well in 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.
PUBLICATIONS


F. Pendino, I. Tarkanyi, C. Dudognon, J. Hillion, M. Lanotte, J. Aradi, E. Ségal-Bendirdjian, ”Telomeres and Telomerase: pharmacological targets for new anticancer strategies?“ (Current Cancer Drug Targets, accepted for publication)
POSTERS and PRESENTATIONS

Posters

Cold Spring Harbor Laboratory Meeting
„Telomeres & Telomerase” (New York, 2005)
“Inhibition of human telomerase by oligonucleotide chimeras, composed of an antisense moiety ad a chemically modified homo-oligonucleotide”

Eurocancer 2005 (Paris)
“Retinoid/arsenic combination therapy of promyelocytic leukaemia: induction of telomerase-dependent cell death”

Annual Meeting of the Hungarian Biochemical Society,
Division of Molecular Biology
“Effect of all-trans-retinoic acid (ATRA) and arsenic trioxide (ATO) combinational treatment on the telomerase expression of acute promyelocytic leukemia cells”(2004)

“Telomerase activity and differentiation in immortal myeloid cell lines” (2002)

“Synthesis and biological characterization of apoptosis inducing lipophyl dAMP derivates” (co-author, 2001)
Workshop on Telomeres and Telomerase  (Madrid, 2003)

“Repression of telomerase by retinoids: a potential new cancer therapy” (co-author)

Presentations

Meeting of the Hungarian Society of Chemotherapy

“In vitro characterisation of oligonucleotide-type telomerase inhibitors containing chemically modified bases” (2004)

“Effects of antisense oligonucleotides against telomerase protein mRNA and telomerase RNA-component in A431 cells” (2001)

PATENTS

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]
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