Approaches for the Inhibition of Human Telomerase Based on the Use of Peptide Nucleic Acids and Hammerhead Ribozymes

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Abstract: The ability of peptide nucleic acids and hammerhead ribozymes, which target different subunits of human telomerase, to efficiently inhibit the enzyme's catalytic activity has been clearly demonstrated in several *in vitro* studies carried out in human immortalized and cancer cell lines. However, the actual efficacy of these molecules still needs to be validated in *in vivo* human tumor models, and such validation appears to be largely dependent on the development of reliable systems for their intracellular delivery.

INTRODUCTION

In the last few years a new generation of anticancer approaches has emerged, based on the knowledge of the molecular mechanisms that contribute to neoplastic transformation and may represent targets for selectively killing cancer cells [1-7].

Human telomerase is a ribonucleoprotein enzyme complex that maintains the telomeric structures at the chromosome termini by adding 5'-TTAGGG-3' repeats [8]. This multisubunit enzyme contains an RNA component, hTR, which provides the template for the synthesis of telomeric repeats [9] and a protein component, hTERT, which possesses conserved reverse transcriptase motifs and catalyzes the synthesis reaction [10] (Fig. (**1**)).

Several lines of evidence indicate that the enzyme is involved in the attainment of immortality in cancer cells and, therefore, may contribute to tumorigenesis and neoplastic progression [11]. The notion that telomerase is reactivated in 80-90% of human cancers [12] has made telomerase a promising target not only for the diagnosis of malignancy but also for the development of novel anticancer treatments.

An evolving understanding of the composition and functions of telomerase has prompted the formulation of distinct rationales for the development of inhibitors [2, 13, 14]. These include antisense-based oligonucleotide inhibitors targeting the RNA component of human telomerase. Physical blockage of hTR by conventional DNA oligomers and phosphorotioate-modified DNA has been reported [15-17]. However, the poor sequence selectivity observed with such compounds has led to a search for second-generation oligonucleotides able to bind complementary sequences with very high affinity. In this context, efficient inhibition of telomerase activity has been obtained by 2' O-methyl-RNAs [18] and peptide nucleic acids [19]. In addition, hammerhead ribozymes have been successfully used to downregulate telomerase activity in human cancer cells [20].

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PEPTIDE NUCLEIC ACIDS

Peptide nucleic acids (PNAs) are DNA/RNA mimics in which the phosphate deoxyribose backbone has been replaced by N-(2-aminoethyl) glycine linkages with the nucleobases attached through methylene carbonyl linkages to the glycine amino group [21] (Fig. (**2**)). The uncharged nature of the PNA internucleotide linkage increases the stability of the PNA/DNA (or PNA/RNA) duplex as a consequence of the lack of charge repulsion between the PNA strand and the DNA (or RNA) strand. The Watson-Crick base pairing rules are strictly observed in hybrids of PNA and nucleic acids [22, 23]. Moreover, PNAs are better at discriminating between base pair mismatches [22] and are less likely to bind to proteins through non-sequence-specific interactions than phosphorothioate oligomers. The lack of electrostatic repulsion between the two strands in a PNA/nucleic acid duplex also leaves the melting temperature (T_m) largely independent of the salt concentration. Again, due to their peculiar chemical structure, PNAs are highly resistant to proteases and nucleases [24].

Their favorable properties have led to the use of PNAs for different applications in oncology. A number of experiments with permeabilized cells, isolated nuclei and also intact cells have demonstrated the potential of PNA in antigene or antisense applications to downregulate the transcription or translation of cancer-related genes. PNA invasion of the DNA double helix to form a stable PNA-DNA hybrid was found to effectively block gene transcription [25-26]. PNAs also efficiently inhibited translation by binding the target mRNA. Such inhibition may be due to physical blocking of the ribosomes during one of the following steps: scanning of mRNA to find the start codon, assembly of the two subunits to initiate the translation, or elongation of the polypeptide chain [27].

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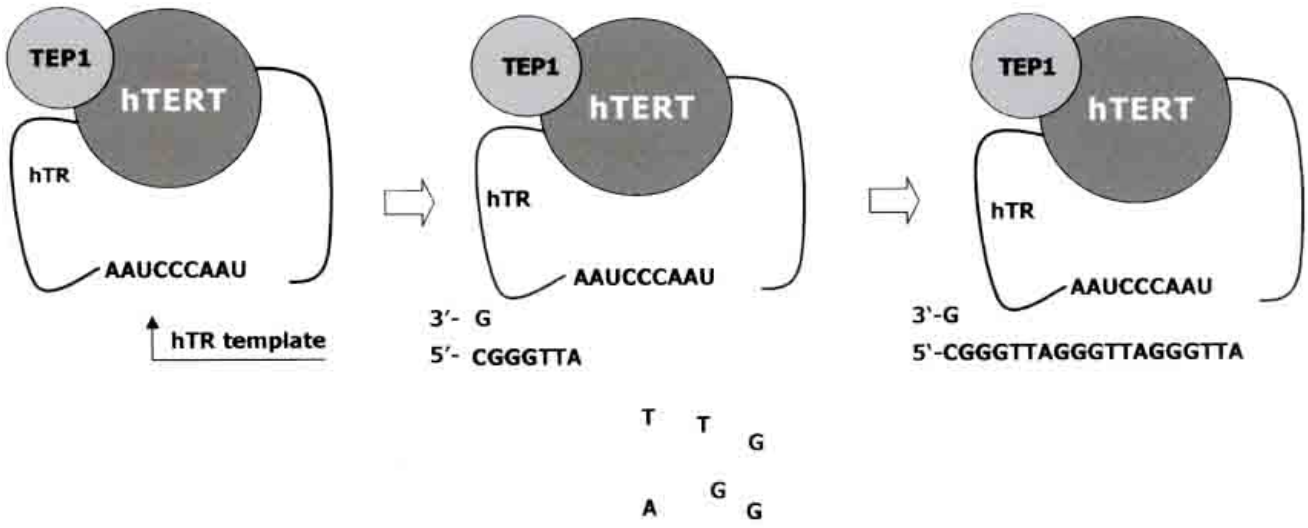

Fig. (1). The human telomerase components and a simplified telomerase reaction mechanism. hTR, human telomerase RNA subunit; hTERT, human telomerase reverse transcriptase; TEP1, telomerase-associated protein. Telomerase action involves, i) primer binding, ii) template-directed nucleotide addition, and iii) translocation.

Naked PNAs are generally not taken up spontaneously by cells [28], with probably the only exception being nerve cells in the intact rat brain [29]. As a consequence, PNAs have to be delivered to cells as conjugates with import peptides [30, 31], by electroporation [32], or as complexes with DNA and cationic lipid [33, 34] or polyethyleneamine [35]. Probably, the most interesting strategy developed thus far for the delivery of PNAs is the one based on the use of so-called "cell-penetrating peptides". Examples include a polybasic sequence from the human immunodeficiency virus (HIV) Tat protein [36], the 16-amino-acid-long peptide corresponding to the third helix of the DNA binding domain of the transcription factor Antennapedia [37], and amphipathic sequences based on signal peptides [38]. To generate cell-penetrating PNA constructs, PNA oligomers have been coupled with cellular transporter peptides, which are able to enter cells in an energy- and receptor-independent manner, thus facilitating the entry of otherwise non-penetrating PNAs [31].

POTENTIAL OF PEPTIDE NUCLEIC ACIDS AS INHIBITORS OF HUMAN TELOMERASE

Successful approaches for the inhibition of telomerase activity have been developed by using PNAs complementary to the RNA component of human telomerase (hTR). Telomerase is an unusually favorable target for inhibition by nucleic acid mimics because the template region of hTR binds to telomere ends and is, therefore, inherently accessible to hybridization by inhibitory oligomers.

Norton *et al.* [19] first demonstrated the possibility to efficiently inhibit telomerase activity in cell extracts of a human immortal primary breast epithelial cell line with PNAs of different length targeting hTR. These authors found that the inhibition was dependent on targeting exact functional boundaries of the hTR template and that it was 10- to 50-fold more efficient than inhibition induced by phosphorothioate oligomers of analogous sequence. Moreover, in contrast to the high selectivity of inhibition by PNAs, phosphorothioate oligomers inhibited telomerase in a non-sequence-selective fashion.

In a successive study carried out by Shammas and coworkers [32], 11-mer and 13-mer PNAs complementary to telomerase RNA were co-electroporated into immortal human cells along with a selectable plasmid. The authors demonstrated that PNA treatment effectively inhibited telomerase activity in intact cells, shortened telomeres, and arrested the proliferation of cells after a lag period of 5-30 cell generations, consistent with the suppression of their

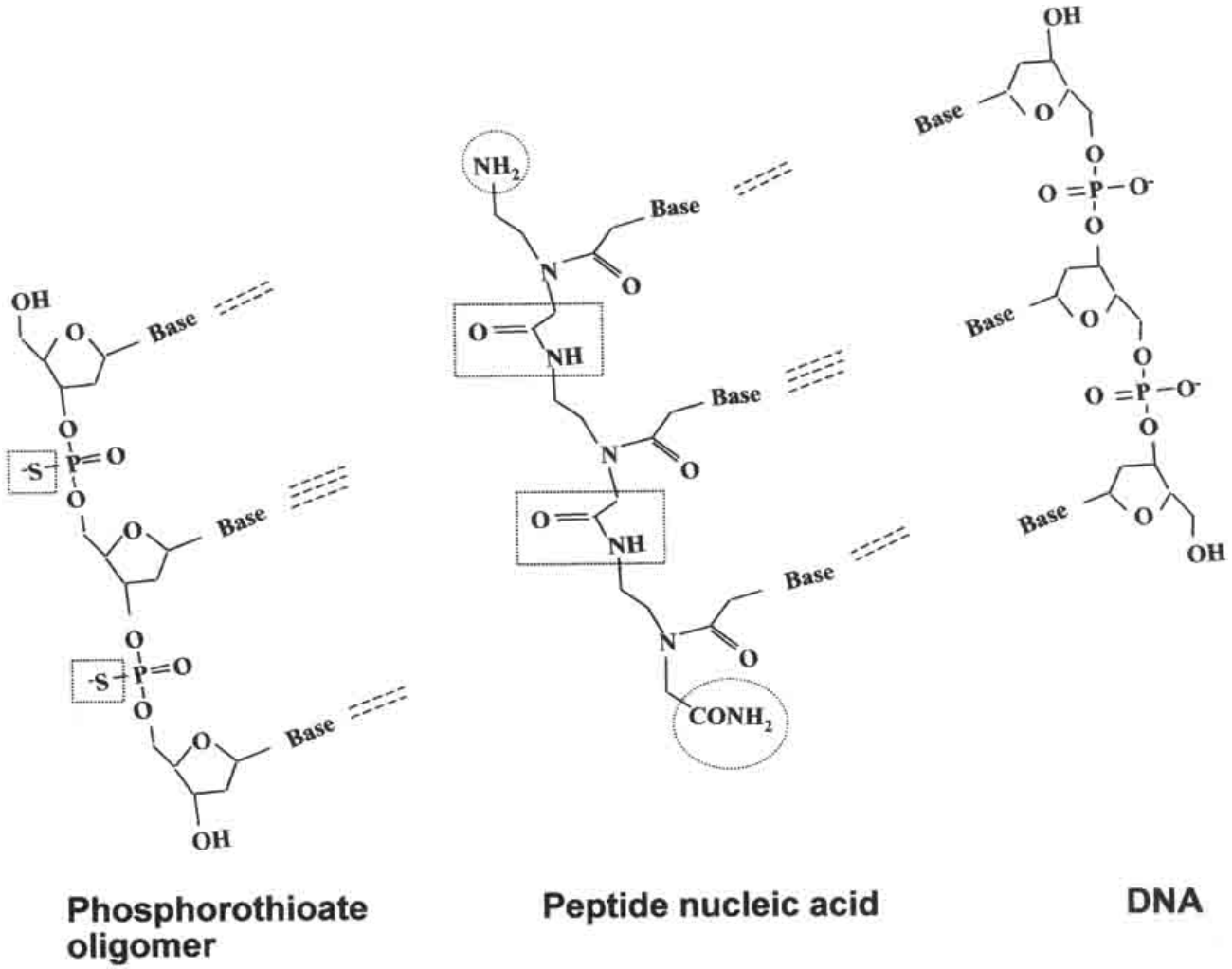

Fig. (2). Chemical structures of peptide nucleic acid, phosphorotioate oligomer and DNA.

immortality. PNA-mediated inhibition of telomerase activity was also demonstrated in the human prostate carcinoma cell line DU145 [39]. To facilitate its uptake into the cells the 13-mer PNA was hybridized with an appropriate DNA oligonucleotide and the PNA/DNA complex was delivered to the cells through the use of cationic lipids. Significant PNA-mediated inhibition of telomerase activity was perceptible starting from day 1 following transfection. Moreover, a marked decrease in telomere length was observed after 76 days of addition of the PNA/DNA complex.

Based on these data, which suggest that PNAs are promising telomerase inhibitors, we evaluated in our laboratory the ability of 11-mer and 13-mer PNAs designed to cover the template and the 5' proximal region of human telomerase, to inhibit the catalytic activity of telomerase in extracts from human melanoma cell lines and surgical specimens [40]. Thirty minutes' exposure to either PNA induced a dose-dependent inhibition of telomerase activity starting from the concentration of 10 nM, although the 13-mer PNA was more efficient than the 11-mer PNA in inhibiting the enzyme, as demonstrated by the lower IC_{50} values (35-70 nM vs 67-95 nM) (Fig. (**3**)). When applied to mildly permeabilized melanoma cells, PNAs continued to inhibit telomerase activity, albeit with markedly higher IC_{50} values than those required for inhibition in purified extracts.

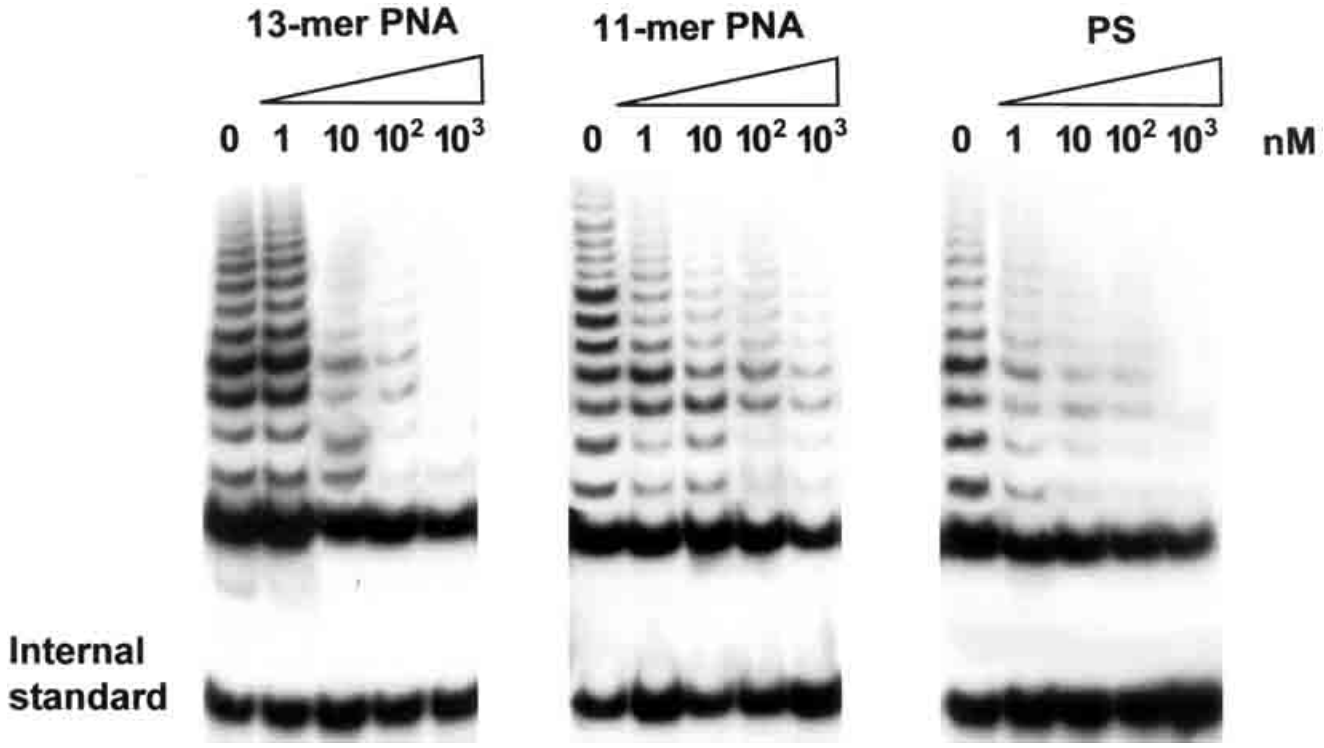

Fig. (3). A representative telomeric repeat amplification protocol (TRAP) assay [40] showing inhibition of telomerase activity induced by 13-mer and 11-mer peptide nucleic acids and phosphorotioate oligomer (PS) targeting hTR in JR8 melanoma cell extracts.

In order to downregulate telomerase activity in intact melanoma cells we developed a strategy for PNA delivery by coupling the 13-mer PNA to the Antennapedia cell-penetrating peptide. The cysteine-containing transport peptide was linked by a disulfide bond to cysteine linker-extended PNA. The disulfide bond is thought to be quickly reduced in the intracellular milieu, leading to the dissociation of the PNA from the carrier peptide, thereby permitting PNA to associate with target RNA. A dose- and time-dependent inhibition of telomerase activity was observed in intact melanoma cells exposed to the cell-penetrating PNA construct for 24-144 h, thus indicating that the chimeric molecule was taken up by cells and that the PNA was able to specifically interact with the complementary RNA sequence (Fig. (**4**)). Internalization of the PNA construct was also confirmed by fluorescence microscopy using a biotinylated chimeric molecule. However, the inhibitory effect on telomerase activity was perceptible at a much higher PNA concentration (in the micromolar range) than those required to induce telomerase inhibition in cell extracts. When treatment with the PNA construct was prolonged for 20 days, cells showed a significantly longer doubling time than untreated control cells. Moreover, fluorescence microscopy analysis revealed the presence of a small but significant percentage of apoptotic cells in PNA-treated samples, in agreement with previous reports indicating apoptosis as one of the possible pathways induced by telomerase inhibition [41]. Interestingly, we failed to observe any shortening of telomere length in PNA-treated cells. However, it should be emphasized that in the cells, telomerase activity was markedly reduced but not completely abrogated.

A somewhat different approach was pursued by Hamilton *et al.* [42], who developed PNAs targeting nontemplate regions of the telomerase RNA which were able to overcome the RNA secondary structure and inhibited telomerase by intercepting the RNA component prior to holoenzyme assembly.

In our laboratory, we recently attempted to develop PNAs targeting the human telomerase reverse transcriptase (hTERT) mRNA. In fact, since the expression of hTERT is the rate-limiting determinant of telomerase activity, the attenuation of hTERT mRNA expression would be an excellent way to regulate the enzyme's activity in tumor cells. However, hTERT mRNA is a more challenging target than hTR for PNAs. In fact, mRNA possesses a complex secondary structure that makes it difficult to accurately predict which target site will be most accessible for hybridization. Moreover, since PNA/RNA hybrids do not support RNase H activity, PNAs only have the possibility to bring about a physical block against the translation machinery because they cannot promote degradation of the

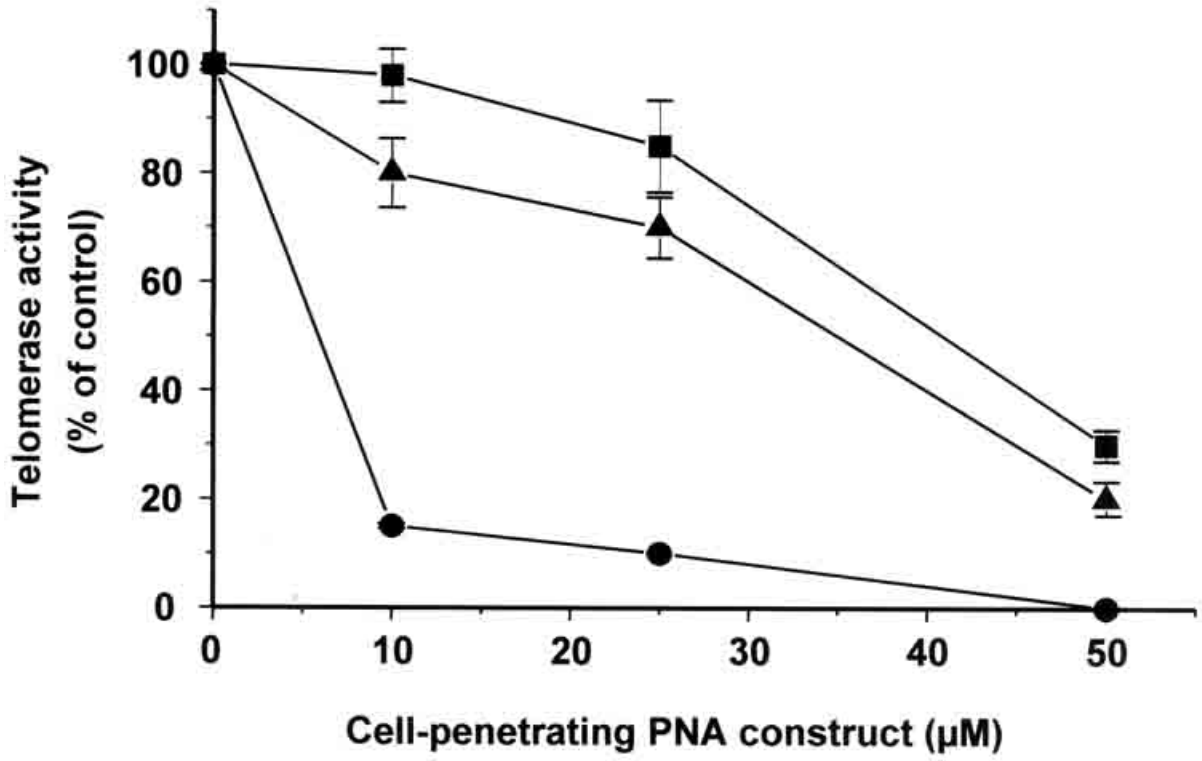

Fig. (4). Quantitation of telomerase inhibition in JR8 cells after 24 (■ ■), 48 (▲ ▲), and 144 (● ●) h exposure to different concentrations of the 13-mer cell-penetrating PNA construct targeting hTR. The effect of the PNA construct on telomerase is expressed as the percentage inhibition of enzyme activity compared to control. The data represent mean values ± S.D.

target sequence. We designed a 15-mer PNA (3'-TCCCGACCGCCGACC-5') directed against one portion (bases 157-171) of the hTERT mRNA. Moreover, in order to deliver it to intact cells, we generated a chimeric molecule by coupling the PNA with the HIV Tat transport peptide (RRRQRRKKR). By using a biotinylated PNA-Tat construct we were able to confirm its efficient incorporation into human ovarian cancer cells. Preliminary experiments carried out by exposing OAW42 human ovarian carcinoma cells to the cell-penetrating PNA construct showed the ability of this molecule to induce a dose-dependent inhibition of telomerase catalytic activity, with IC_{50} values ranging from 7.2 μM (for a 16-h exposure) to 2.2 μM (for a 24–h exposure) (unpublished results).

HAMMERHEAD RIBOZYMES

Ribozymes are small RNA molecules which possess specific endonucleolytic activity and catalyze the hydrolysis of specific phosphodiester bonds, resulting in the cleavage of the RNA target sequences [43]. In nature, ribozymes catalyze sequence-specific RNA processing. The specificity is determined by Watson-Crick base-pairing between ribozymes and nucleotides near the cleavage site of the target RNA. By altering substrate recognition sequences, several intramolecular *cis*-cleaving ribozymes can be designed to cleave any RNA in *trans*. Theoretically, these *trans*-cleaving ribozymes can be designed to cleave any RNA species in a sequence-specific manner by incorporating the flanking sequences complementary to the target. After the cleavage reaction the substrate is accessible to ribonucleases, a step that guarantees its permanent inactivation and offers a considerable advantage over the simple physical blockage obtained with complementary oligodeoxynucleotides.

Several catalytic domains derived from naturally occurring ribozymes have been identified, the most common among which being the hammerhead and hairpin structures from small plant pathogenic RNAs (satellite RNAs, viroids and virusoids) [43]. These RNA catalytic motifs have received much attention in view of their potential usefulness due to their inherent simplicity, small size and ability to be incorporated into a variety of flanking sequence motifs without changing site-specific cleavage capacities [44]. In particular, the hammerhead ribozyme consists of a highly conserved catalytic core, which cleaves substrate RNA at NHH triplets 3' to the second H, where N is any nucleotide and H is any nucleotide but guanidine [45] (Fig. (**5**)). In addition to the catalytic core, a particular cleavage site in a target RNA can be specifically recognized by creating, in the hammerhead ribozyme arms, flanking sequences complementary to the specific target RNA molecules.

As for antisense oligonucleotides and PNAs, one of the major limitations to the therapeutic use of hammerhead ribozymes is the problem of delivery. There are two main ways to deliver the ribozyme to its cellular target RNA

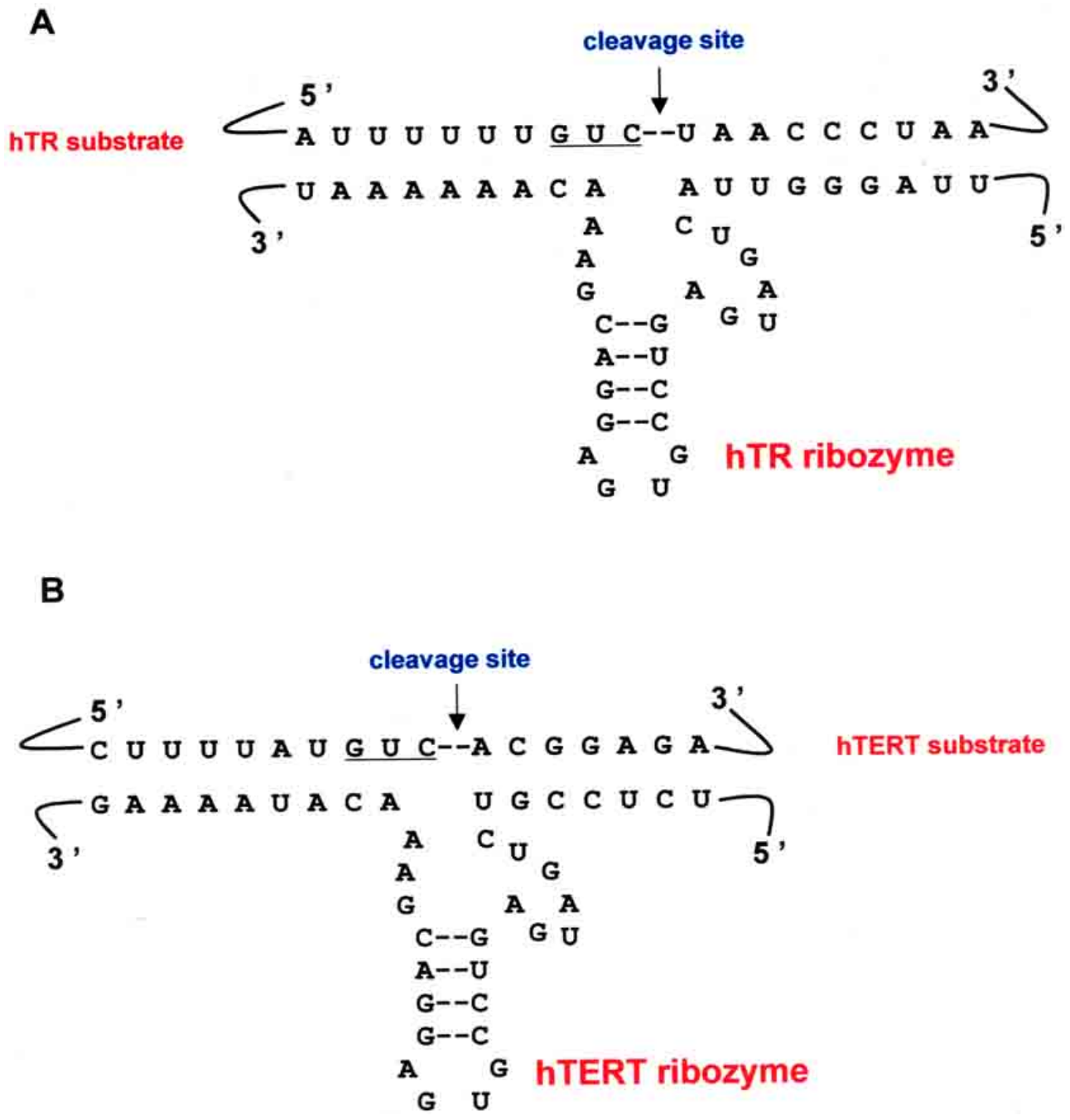

Fig. (5). Schematic representation of hammerhead ribozymes directed against hTR (A) or hTERT mRNA (B) annealed to synthetic substrates. The cleavage sites on the substrates are indicated.

within the cells. One is exogenous ribozyme delivery, in which presynthesized ribozymes are introduced directly into the cells with the aid of cationic liposome-mediated transfer [46]; the other is endogenous delivery, i.e., the intracellular transcription of a ribozyme coding sequence accomplished by transfection/infection of ribozyme-producing vectors into cells. By using both approaches a number of studies on experimental human tumor models have shown the possibility to obtain efficient inhibition of the expression of several cancer-associated genes through the use of hammerhead ribozymes [5-6].

POTENTIAL OF HAMMERHEAD RIBOZYMES AS INHIBITORS OF HUMAN TELOMERASE

Kanazawa *et al.* [20] first developed a hammerhead ribozyme that was engineered to cleave the 3' end of the GUC_{46} located at the end of the telomerase template [47]. This ribozyme contains a catalytic domain with 10bp flanking nucleotides that are complementary to the sequences 5' and 3' at the target site (Fig. (**5A**)). *In vitro* the ribozyme induced cleavage of a synthetic RNA substrate obtained by cloning a portion of the RNA component of human

telomerase. Moreover, when added to cell extracts from two hepatocellular cacinoma cell lines (HepG2 and Huh-7), the ribozyme inhibited telomerase activity in a dose-dependent manner.

In order to downregulate telomerase activity in intact human tumor cells we developed in our laboratory strategies for intracellular delivery of the above-mentioned ribozyme targeting hTR [46, 48]. We first generated a cationic liposomal complex by mixing the hammerhead ribozyme with DOTAP, and a significant reduction of telomerase activity was observed in JR8 human melanoma cells exposed for 48 h to the exogenously delivered ribozyme. However, the inhibitory effect was detectable at a much higher ribozyme concentration than those required to induce telomerase inhibition in cell extracts. This difference might be due to a number of factors including the inactivation of the ribozyme by endogenous nucleases and the low endocytotic activity of the cellular model. In a further step, we inserted the ribozyme sequence into a plasmid expression vector, under the control of the CMV promoter, and transfected JR8 human melanoma cells with it. We were able to select ribozyme transfectants successfully expressing the ribozyme and characterized by reduced telomerase activity and a decreased level of telomerase RNA expression compared with control cells (Fig. (**6**)). Moreover, ribozyme transfectants grew more slowly than parental cells and also expressed an altered morphology with a dendritic appearance in monolayer culture. A small but significant fraction of the cell population also expressed an apoptotic phenotype. However, no telomere shortening was observed in these clones even after a prolonged period (50 days) of growth in culture.

Yokoyama *et al.* [49] also synthesized three hammerhead ribozymes targeting the 3' end of the GUC sequences at 44-46 (the template region), 178-180, and 323-325 from the 5' end of telomerase RNA [47]. In a cell-free system all the ribozymes efficiently cleaved the RNA substrate. However, when the ribozymes were introduced in intact endometrial carcinoma Ishikawa cells, only the ribozyme targeting the template region (which is essentially the same ribozyme proposed by Kanazawa [20] and developed in our laboratory [46, 48]) was able to diminish telomerase activity. The ribozyme sequence was then inserted into an expression vector and another endometrial carcinoma cell line, AN3CA, was transfected with the vector. Ribozyme-expressing clones obtained after *in vitro* selection showed reduced telomerase activity and telomerase RNA expression. In some of these clones a marked reduction of telomere length was observed. However, even after 30 passages *in vitro* these cells still maintained their ability to proliferate.

Recently, Yokoyama *et al.* [50] evaluated the potential of hammerhead ribozymes targeting the RNA messenger of telomerase catalytic subunit hTERT for the inhibition of human telomerase activity. For this purpose seven ribozymes were designed to target different sites of hTERT mRNA and delivered to endometrial carcinoma cells by cationic lipid-mediated transfer. Only ribozymes 13 RZ and

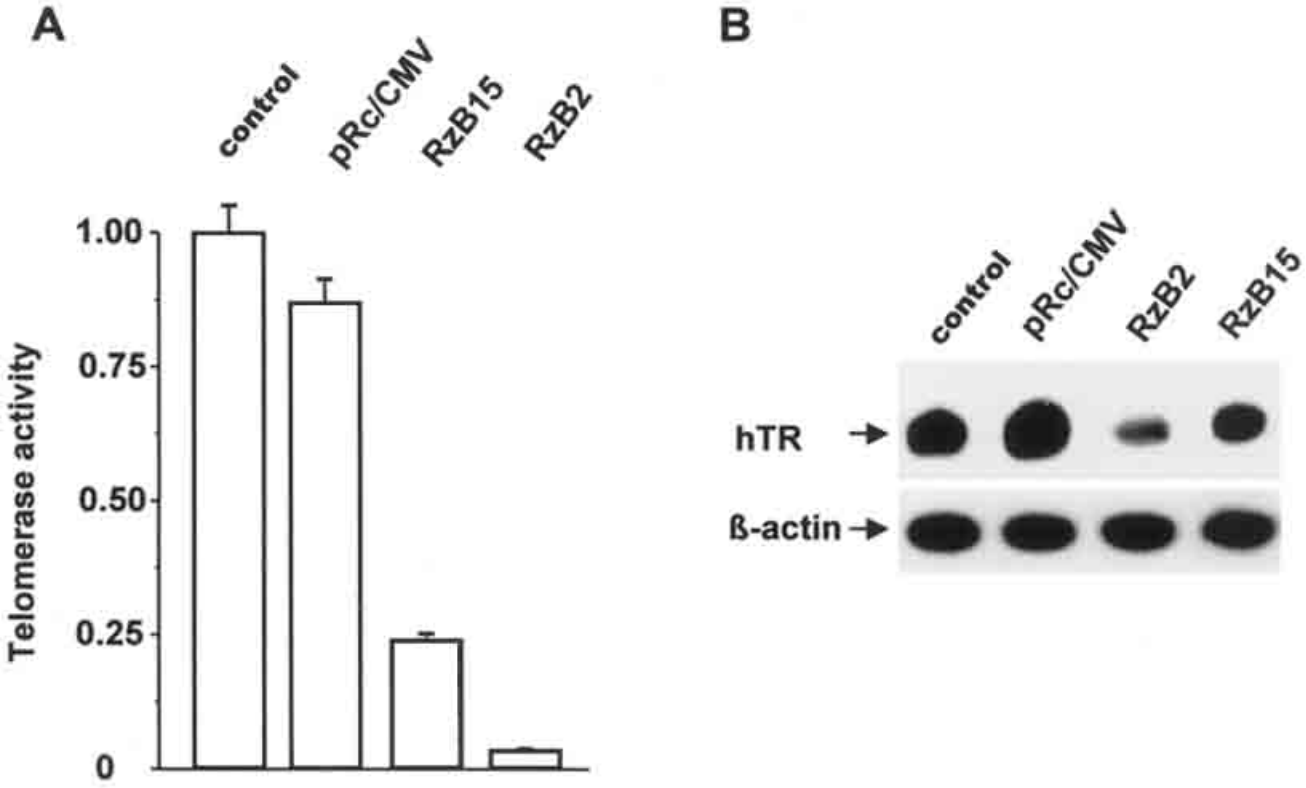

Fig. (6). A) Quantitation of telomerase activity in JR8 cells (control), vector-transfectant clone (pRc/CMV) and clones transfected with the ribozyme targeting hTR (RzB2, RzB15). (B) hTR expression as detected by RT-PCR in vector-transfectant and ribozyme-transfectant clones after 45 days of growth in culture.

3950 RZ, designed to the 3' end of GUC_{13} and GUC_{3950} in the hTERT mRNA (GeneBank, accession n. AF015950), were able to inhibit telomerase activity in intact cells. A stable transfection study carried out by cloning the ribozyme sequences into expression vectors and transfecting endometrial carcinoma cells with them confirmed the ability of only one ribozyme (13 RZ) to suppress telomerase activity.

More recently, Ludwig *et al.* [51] developed a hammerhead ribozyme able to cleave hTERT mRNA within the T-motif at the 3' of the C at position 1744 (GeneBank, accession n. AF015950) *in vitro* and to diminish the expression of target mRNA in intact cells (Fig. (**5B**)). Specifically, the ribozyme attenuated telomerase activity in stable transfected clones of the immortal, telomerase-positive human breast epithelial cell line HBL-100 and the breast cancer cell line MCF-7 and in adenovirus-infected mass cultures of HBL-100. Moreover, in ribozyme-transfected clones the decline of the enzyme's catalytic activity was accompanied by telomere shortening, inhibition of net cellular growth and induction of apoptosis. Again, clones with reduced telomerase activity showed an increased sensitivity to inhibitors of topoisomerase II such as doxorubicin, etoposide and mitoxantrone.

PERSPECTIVES

The results reported in the literature to date indicate that PNAs and hammerhead ribozymes can specifically downregulate the expression/activity of human telomerase and may possess potential for cancer therapy. Nevertheless, many issues remain to be addressed before these molecules can be added to the anticancer armamentarium. In fact, the actual efficacy of PNAs and ribozymes has only been determined in *in vitro* tumor cell systems and needs to be validated in *in vivo* experimental tumor models. Obviously, such validation will be largely dependent on the development of reliable systems for intracellular delivery of PNAs and ribozymes.

Even though telomerase is considered a promising target for new anticancer interventions, it has not yet been clarified how telomerase inhibition affects the proliferative capacity of tumor cells. In fact, specific telomerase inhibition in human tumor cells leading to telomere shortening and cell death has been demonstrated in a still limited number of papers. The lack of telomere shortening following telomerase inhibition that was observed in some studies [40, 41, 46, 48] might be tentatively explained by the emergence of cell populations characterized by the presence of alternative lengthening of telomeres (ALT) mechanisms, which are responsible for the maintenance of telomeres -through recombination events [52]- and have been demonstrated to be present in a small minority of tumors. Recent results obtained in different laboratories [53, 54] point to the possibility that ALT mechanisms and telomerase coexist in the same tumor cell. Results obtained in hybrid somatic cell clones generated by fusion of ALT-expressing cells with telomerase-positive cells indicate that in such hybrids telomerase is maintained and ALT is repressed, even though the factor(s) responsible for ALT repression are still to be identified. However, it cannot be excluded that ALT repression in hybrids is indirectly mediated by telomerase, probably in concert with other telomerase-associated factors [53]. Such evidence would suggest that in particular cell sytems the marked and prolonged inhibition of telomerase activity (for example as a result of PNA or ribozyme treatment) is responsible for the reactivation of ALT mechanisms in these cells.

In various telomerase-positive tumor cell models induction of apoptotic cell death has been observed after a few days of treatment with telomerase inhibitors [40, 41, 46]. The results of these studies cannot be explained by the classical model which predicts that long-term exposure of tumor cells to telomerase inhibitors should induce telomere shortening after a certain number of rounds of cells division (the number being dependent on the initial telomere length) and growth arrest. In fact, in the aforementioned studies it is unlikely that cell death was related to telomere erosion since the cells would not have undergone enough divisions to significantly shorten their telomeres. Interference with telomerase activity might, therefore, affect aspects of the control of cell proliferation and apoptosis, other than telomere length. Recent evidence suggests that telomeres normally exist in a capped state, but may switch to an uncapped state. The appropriate response to the uncapping of a telomere is action by telomerase to protect the telomere so that cell cycling can resume [55]. Based on these findings it could be hypothesized that, when there is a marked inhibition of telomerase activity, the enzyme is no longer able to protect the telomere and cells can die through a mechanism independent of telomere length. Such results would suggest that abrogation of telomerase activity may also affect cell proliferation through pathways that are not dependent on telomere erosion, and indicate that the availability of effective telomerase inhibitors has important implications for our understanding of the role of telomerase activation during human oncogenesis.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

hTR = Human telomerase RNA

hTERT = Human telomerase reverse transcriptase

PNA = Peptide nuclei acid

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