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Review on telomerase activity in metastasis and proposal of amino-modified polystyrene-antisense human RNA nanoparticles in killing metastatic cells.

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Abstract: Telomeres protect chromosomes from losing base-pair sequences at their ends. Part of telomeres is lost during each cell division. When the telomere becomes too short, the chromosome can no longer replicate. Telomerase, by reverse transcription, maintains telomere length and stability in cancer, gonadal and hemopoietic stem cells. During telomerase maturation, the RNA component is transferred to the cytoplasm where it recruits proteins and the mature complex is then re-imported into the nucleus. Telomerase activity in cancer cells is 10-20 times greater than that in normal cells.

Amino silica nanoparticles are transport carrier for antisense human telomerase RNA (hTR) to cytoplasm where hTR binds to telomerase mRNA leading to its degradation. Sometimes anti-telomerase leads to growth arrest but not to cancerous cell death.

It is proposed that amino polystyrene (NH_2PS) can replace amino silica nanoparticles and carry antisense hTR to the cytoplasm. After the binding of the antisense hTR to the mRNA of telomerase, NH_2PS nanoparticles will be released. These nanoparticles inhibit the mammalian target of rapamycin (mTOR) signaling, leading to G2 cell cycle arrest of all cells exhibiting activated mTOR signaling including cancer cells. Due to the absence of mTOR signaling, macrophages resist to NH_2PS toxicity. Besides, NH_2PS inhibits angiogenesis and proliferation of tumour cells.

Keywords: Antisense human telomerase RNA (hTR), amino polystyrene nanoparticles, mammalian target of rapamycin (mTOR), tumor cells, growth arrest.

Introduction

Membrane proteins are targets for immune surveillance against cancer cells. These cells can escape the antigen-specific T cells immune surveillance by losing major histocompatibility complex class I and II antigens. They can also undergo mutation leading to chemotherapy resistance in addition to overproduction of some proteins that render anti-cancer therapy ineffective. The p-glycoprotein in cancer cells can pump the chemotherapeutic agent out of the cells, or a mutation in the carrier that transports the drug across the cell wall may inhibit the protein and the entrance of the drug. Moreover, cancer cells can repair its DNA breaks, which together with tumor-derived soluble factors, facilitate the metastatic spread [1].

Human telomere is a TTAGGG sequences repeating around 2,500 times at each end of chromatid. It protects the chromosome from enzymatic end-degradation. Telomeres get shortened with each mitosis until a certain length when the chromosome can no longer replicate. Telomerase, being present in 85 to 90% of cancer cells, maintains the telomere length by reverse transcription.

Telomerase activity has been found in the cytosolic fraction of tumour cells by means of cell fractionation through solubilization in trifluoroethanol or by a two-dimensional electrophoresis of whole-cell, membrane and soluble extracts [2]. Telomerase activity has been demonstrated in 75% of oral carcinomas, 80% of lung cancers, 84% of prostate cancers, 85% of liver cancers, 93% of breast cancers, 94% of neuroblastomas, 95% of colorectal cancers, 98% of bladder cancers and in cell lines transformed by oncogenic simian virus 40 or human papillomavirus types 16/18 [3].

Telomerase is activated during cell immortalization and tumorigenesis. Normal germ-line cells and hemopoietic stem cells are also telomerase-positive but to a lesser extent [4].

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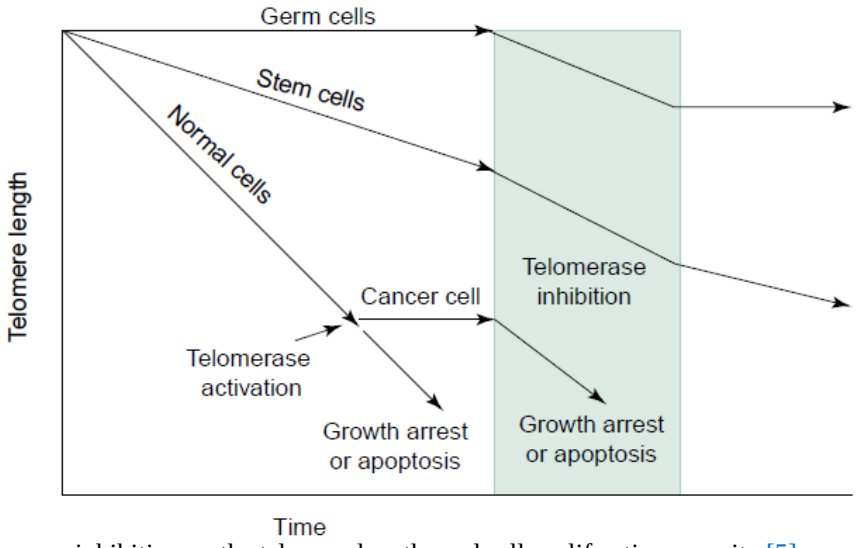

Figure 1. Effects of telomerase inhibition on the telomere lengths and cell proliferative capacity [5].

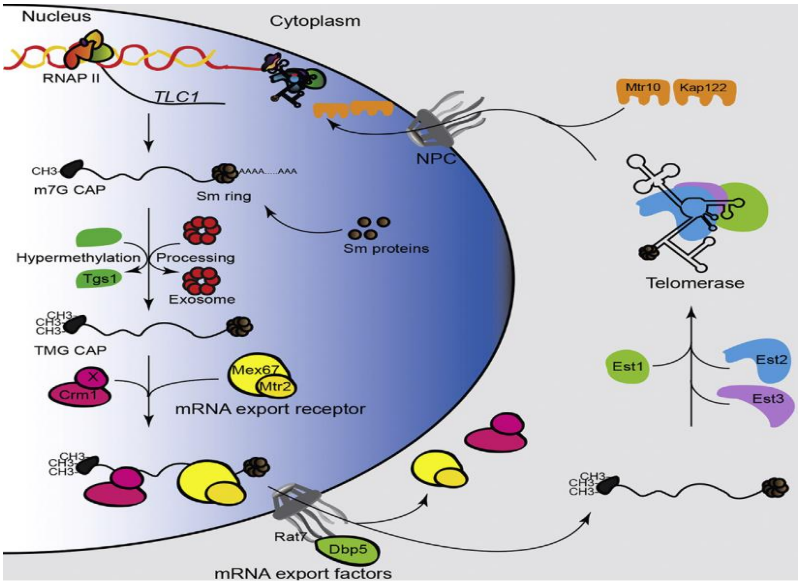

Figure 2. Part of TLC1 life cycle is in cytoplasm [6].

Anti-telomerase is useful in cancer therapy and four molecular approaches have been developed for this concern. 1) Antisense hTR oligonucleotides, 2) hammerhead ribozymes (hTR),3) small molecules (G-quadruplex stabilizers, combinatorial libraries, compound collections) and 4) catalytic human telomerase reverse transcriptase component (reverse transcriptase inhibitors, dominant-negative hTERT, immunotherapy) [7,8].

Antisense hTR oligonucleotide was first reported in 1995, and worked against the first 185 nucleotides of the hTR molecule. It aims to attenuate the tumor cell viability. However, its action is telomere length-dependent, if the tumor has rather long telomere, the antiproliferative effect is not obvious. It is the most effective anti-telomerase due to the complementary matching between the Watson-Crick base pairing.

Hammerhead ribozymes are small RNA molecules cleaving their RNA substrate in a sequence-dependent manner at GUX motifs. They work against hTER and reduce the telomerase activity. It delays the doubling of tumor but there is no telomere shortening.

G-quadruplex stabilizers inhibits telomerase by hindering the enzyme from elongating the 3' overhang. Telomeric DNA ends in a single-stranded 3'-G-rich overhang of 150 - 200 bases in length. The 3' overhang is guanine rich and can form G-quadruplex secondary structures under physiologic ionic conditions. Drugs stabilizing these quadruplex will inhibit the enzyme.

The gene for the catalytic subunit of telomerase, hTERT, was discovered in 1997. A dominant negative mutant of the hTERT gene is an altered gene product that acts antagonistically to the wild-type allele. These mutations usually result the in inactivation of the telomerase function.

Antisense hTR therapy is effective in hepatocellular carcinoma and gastric cancer [9,10].

It forms complex with RNA-induced silencing complex (RISC) and binds to mRNA which is then cleaved by slicer. RISC is a ribonucleoprotein incorporating small interfering RNA or micro-RNA. The single strand hTR acts as a template for RISC to recognize the complementary messenger RNA. The protein Argonaute in RISC activates and cleaves the mRNA (Figure 3). This RNA interference is used in gene silencing and defense against viral infections [11].

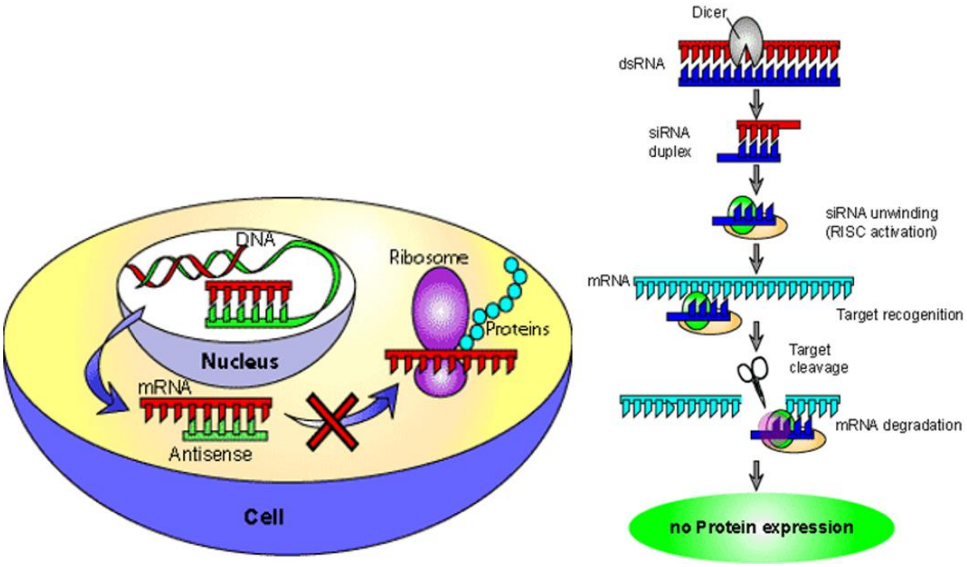

Figure 3. Mechanism of action of antisense RNA [11]

Is anti-telomerase effective in tumors without p53 gene mutation?

Telomerase activity in tumors is often associated with mutation in p53 which is a tumor suppressor often lost or inhibited in cancer cells. Loss of p53 function accelerates acquisition of telomerase activity. However, some tumor types, like breast cancer, melanomas and thyroid cancer, retain wild-type p53 function mediating a growth arrest response to telomere erosion. In other words, anti-telomerase is effective in treating cancer cells with both wild type p53 or mutated p53 gene. Telomere erosion induced by the expression of a dominant-negative (DN) hTERT resulted in delayed onset of growth arrest. Although, long-term anti-telomerase treatment is a possible solution, resistant tumor clones may emerge [12].

Tumor cell apotosis as a result of mTOR inhibition [13]

Rapamycin is a macrolide antibiotic produced by Streptomyces hygroscopicus. The intracellular receptor of rapamycin is FKBP12.Rapamycin-FKBP12 complex interacts with the mammalian target of rapamycin (mTOR) and inhibit its signaling to downstream targets. mTOR masters the cellular metabolism and controls cell apoptosis.

mTOR is a serine/threonine kinase which signals to downstream effectors. With growth factor and nutrients, mTOR is activated through the insulin receptor or insulin-like growth factor receptor pathways, involving the activation of PI3K (phosphatidylinositide-3-kinase) and the Akt/PKB mediated phosphorylation.

OncemTOR increases mRNA translation inhibits autophagy and inhibits apoptosis by inhibiting Rapamycin. Rapamycin, on the other hand, induces cell apoptosis and has antiproliferative effects on T cells.

Advance in nanotechnology:

Nanoparticles (NP) can interact with cells and serum molecules due to their small dimension, surface charge and surface functionalization (figure 4). Half uptake is characterized by a sub-diffusive behavior, and half uptake shows an active transport. 100 nm-size particles are shown to be internalized by cells much more efficiently than microparticles, which are taken up primarily by phagocytosis. And the uptake of 100 nm particles is 2.3-fold greater than that of 50 nm particles [14-16].

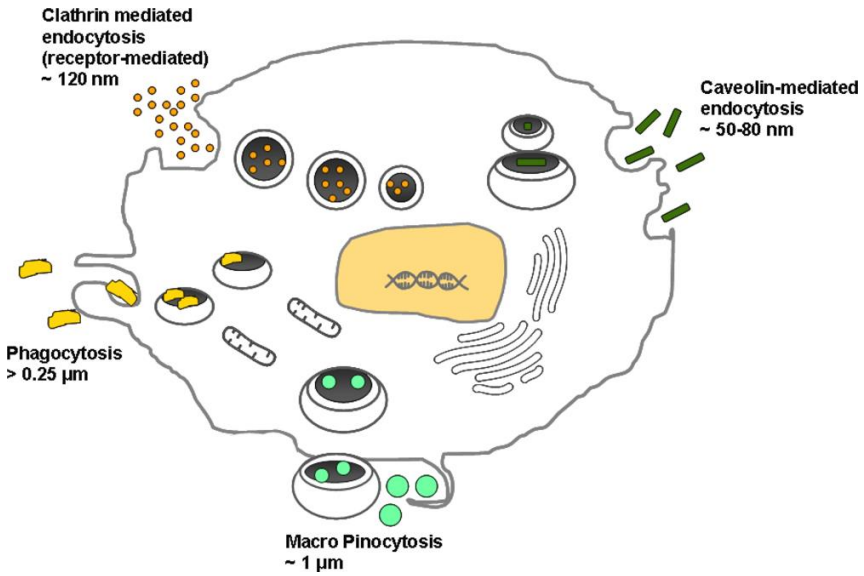

Figure 4. Cell entry mechanisms: the various uptake mechanisms of macromolecules and nanoparticles into cells are indicated according to size [17].

Polystyrene NP can enter and pass through the endothelial cells as shown in (Figure.4). These NP are labelled with fluorescence and the kinetic uptake, endocytic pathway and intracellular trafficking can be studied by confocal microscopy. The cellular uptake of NPs in serum-free medium follows saturation kinetic. The uptake rate is reduced in 10% fetal bovine serum-enriched medium because the protein adsorption on NP surface can affect cellular uptake kinetics, but not active uptake mechanisms. Moreover, Polystyrene NP does not degrade inside cell and can be synthesized in different size with different surface functionalization, making it a platform to study bio-nano interactions [16].

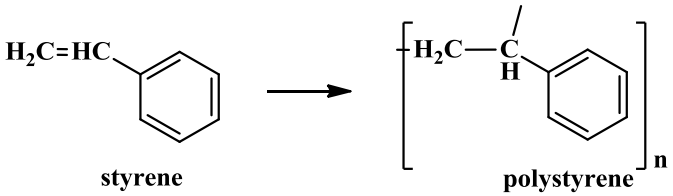

Figure 5. Chemical structure of styrene and polystyrene

Positively charged amino-modified polystyrene nanoparticles (NH_2PS) induce cell death.

NH_2PS can be synthesized by cross-linking reaction between the polystyrene backbone and amino functional moiety under ultradilute conditions. The polymers are characterized by gel permeation chromatography, nuclear magnetic resonance, dynamic light scattering and atomic force microscopy. The amino moiety in the nanoparticle is confirmed by its reaction with trimethylacetyl chloride.

Lung cancer epithelial A549 cells were incubated with different concentration of NH_2PS for different exposure times in cell culture plate medium. In biological fluids nanoparticles can adsorb proteins to form biomolecular corona. On cellular internalisation, NH_2PS accumulate in lysosomes causing swelling and membrane damage. Proteolytic enzymes leak out and trigger apoptosis together with mitochondrial damage and production of reactive oxygen species.

NH_2PS lower than 100 µg/ml causes cell cycle arrest, but not cell death in lung epithelial cells. Monitoring cell numbers revealed that at these lower doses, cell proliferation took place during the first 24 hours. After that, the cell number becomes static suggesting an arrest in cell cycle progression. Cell death remains low for several days. Nanoparticle uptake is affected by cell cycle progression. It can trigger different pathways depending on the exposure conditions and the dose accumulated [17,18].

Amino-polystyrene-antisense human telomerase RNA (NH_2PS-antisense hTR) nanoparticles in treating metastasis.

An antisense oligonucleotide carrier based on amino silica nanoparticles has been documented and is shown to inhibit the cancer growth [18-20]. If amino-polystyrene (NH_2PS) nanoparticles are used instead of amino silica nanoparticles, the cellular uptake of bio-conjugate will be favored. After intravenous administration, the NH_2PS-antisense hTR bio-conjugate will enter the blood stream to the vascularized metastatic tissue, and then to the tumor cell [21].

The cytotoxicity of amino-polystyrene NP is higher than silica. Both NPs are easily synthesized

and are good carriers of antisense hTR but autophagy with amino-silica NP is detected only at high concentrations (above 250 µg/ml) while in the case of amino-polystyrene NP, tumor cell growth arrest is obvious at 100 µg/ml level in tissue culture medium [22,23]. In addition, amino-silica NP has aggregation problem which can be reduced by adding inert functional groups, such as methyl phosphonate, to the surface [20].

NH_2PS-antisense hTR will be administered intravenously only to patients with metastasis. Inhaled route will release the NH_2PS prematurely due to the action of cytochrome P450 CYP2A13 in the respiratory tract. Gastric acid will do the same thing by converting amide to amine.

The level of NH_2PS of 100 µg/ml is the nanoparticle concentration in the cell culture medium. It should be equivalent to the blood level of patients which is the culture medium for the metastasis. Excess antisense hTR will be metabolized by body fluid nucleases.

NH_2PS-hTR

NH_2PS protects antisense hTR from degradation by DNase I. Antisense hTR will recognize and bind to the intracytoplasmic complementary mRNA sequence . After binding, NH_2PS nanoparticles will be released and kill the tumor cell by inhibiting the mTOR. Fluorescent NH_2PS particles (Figure.5) are made by mini-emulsion polymerization with N-(2,6-diisopropylphenyl) perylene- 3,4- dicarboximide and can be traced by confocal fluorescent microscopy [24].

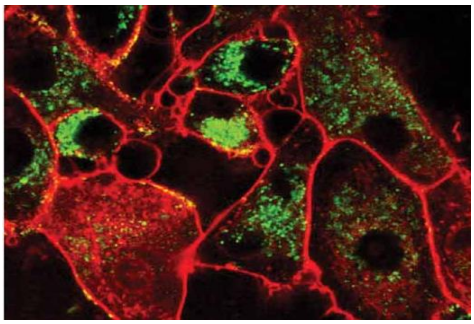

Figure 6. Confocal fluorescent microscopy of HeLa cells after the uptake of amino functionalized particles (green) [25].

NH_2PS-hTR bio-conjugate development:

1) Dosage: Dosage of NH_2PS-hTR bio-conjugate should be adjusted according to patient's blood volume and to tumor growth rate. Low dose causes cell cycle arrest but higher dose is required for acute leukaemia because of the faster spread of malignancy. Sterilization and pancytopenia should be monitored as both gonads and bone marrow stem cells have telomerase activity.

2) In-vitro study: The positively charged NH_2PS nanoparticles is synthesized by a water-in-oil microemulsion method. NH_2PS-antisense oligonucleotides complexes are formed by electrostatic interaction, and their cellular uptake is visualized using fluorescein isothiocyanate -labeled oligonucleotides and NH_2PS doped with rhodamine 6G isothiocyanate (RITC) as fluorescent signal indicators. As both he antisense oligonucleotide and the NH_2PS nanoparticles are toxic to the tumor cell in-vitro, the proliferation and the survival of Hela cells and A549 lung adenocarcinoma cells will be much decreased. The antisense inhibition efficiency of NH_2PS-hTR bio-conjugate can be evaluated using 3,4,5-dimethylthiazol- 2,5-diphenyl tetrazolium bromide (MTT) assay and western blot analysis[22].

3) Animal study: NH_2PS-antisense hTR nanoparticles are injected into HepG2 tumor- bearing nude mice with a dosage according to the blood volume of the mice. The response to this bio-conjugate is assessed by tumor volume. The telomerase activity, cell growth curves, proliferating cell nuclear antigen expression, cell cycle distribution and cell apoptosis are detected by telomere repeat amplification protocol, MTT assay, immunofluorescence, flow cytometric analysis and transferase-mediated nick end labeling [9].

4) Human study: Phase II trial on patients with metastatic carcinoma or leukaemia is suggested. Higher dose for acute leukaemia cases and lower dose (<100 µg/ml using blood volume for calculation) for relatively slow-growing malignancy. 100µg/ml is the level originally described for NH_2PS to cause cancerous cell growth arrest.

40 patients or more are enrolled to determine the drug efficacy and safety. There are 10 patients per group and 4 groups in total, using multi-dose and parallel design.

NH_2PS-antisense hTR nanopaticle will be given intravenously once daily as the elimination half-life of polystyrene nanoparticles in rat model is around 13.8 hours [26]. Tumor size, clinical response and the side effect of pancytopenia will be monitored.

Inhalation route is not recommended because the enzymes present in respiratory tract, like cytochrome P450 2A13, will degrade the NH_2PS-antisense hTR pre-maturely and release the cellular toxin NH_2PS nanoparticles .

To assess the efficacy of NH_2PS-antisense hTR nanoparticles in tackling metastasis, Kaplan-Meier survival curve is used. It estimates the survival function from life-time data by measuring the fraction of patients living for a certain time after receiving NH_2PS-antisense hTR gene[27].

The data is right-censor if a patient withdraws from a study, is lost to follow-up, or is alive without event occurrence at last follow-up. Status at last observation and the time to death are recorded for each patient [27, 28].

Log-rank test compares the survival distributions of two samples on 2 different treatments. This non-parametric test is used when the data are right skewed and censored. New NH_2PS-antisense hTR treatment is compared with the cytotoxic treatment when the measurement is the time to death in patients with metastasis.

Different response to NH_2PS-antisense hTR nanoparticles

We can assess which attributing factors will have the best response to treatment using Cox regression analysis**.** It is a proportional hazards model that relates the time elapsed before death occurs to one or more covariates. The effect of each covariate is multiplicative with respect to the hazard rate.

Discussion

As compared to traditional chemotherapy, the proposed bio-conjugate is relatively selective. Although normal tissue, like mesenchymal , gonadal, bone marrow and other stem cells also have cytosolic telomerase activity, It is 10-20 times less than that of metastatic cells.

By adjusting the blood level of the proposed bio-conjugate and observing the metastatic tissue growth, we hope to achieve cell growth arrest without too much side effects. We should monitor the complete blood picture, liver function and renal function tests regularly.

Just simple administration of antisense hTR nanoparticles is not useful since they will be degraded by nucleases in body fluids. Furthermore, it is not effective against tumor with long telomere. There is a significant time lag between the time of nanoparticle administration and the time of clinical response.

Target therapy is another option of molecular therapy but it is only available for certain tumors. Tumors can mutate and become resistant to target therapy after some time. So far, telomerase RNA mutation is relatively uncommon so that the binding of telomerase RNA to antisense hTR nanoparticles is ensured.

After a certain period, the metastatic tumors will be resistant to the traditional chemotherapy by different mechanisms, as stated above. This is not seen in the tumor cell killing by amino-polystyrene nanoparticles. So the good point of the proposal is the **'sure'** killing of metastatic cells
The challenge will be how to obtain the correct dosage of this bio-conjugate. Just like other medicines, it can be **'a curative or a toxic drug'**, depending on the dosage.

To study the toxicity of this bio-conjugate, we need to study its metabolism and that of amino-polystyrene nanoparticles. We use the lowest possible bio-conjugate dosage to avoid liver damage even if this organ has good regenerative power, with the ability to revert the bio-conjugate toxicity. The metabolism of isolated antisense hTR nanoparticles is not be considered since they will soon be metabolised by body fluid nucleases.

Conclusion

The proposed NH_2PS-antisense hTR nanoparticles bio-conjugate uses the principle of differential cell killing. Metastatic cells will be killed more efficiently than normal tissue as telomerase levels are higher in the immortal tumor cells. The critical point in this design is the bio-conjugate dosage, which should start from low dosage and then stepped up, aiming at metastatic cell growth arrest but not immediate cell killing.

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