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Reporter System for Detection of G-Quadruplexes in the Human Telomerase Reverse Transcriptase Gene Promoter Region

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Abstract—In 80-100% of cases, transformation of human somatic cells into tumor cells is associated with the increased expression of the catalytic subunit of telomerase reverse transcriptase (hTERT). The *hTERT* gene transcription inhibition in tumor cells may become one of the approaches to antitumor therapy. The *hTERT* promoter contains a G-rich region with length of 68 nucleotides, which is capable of forming G-quadruplexes (G4) under certain conditions *in vitro*. It is known that G4s interfere with activity of the human RNA polymerases. Thus, the G4 structure stabilization in the promoter could be considered as a possible strategy to reduce *hTERT* expression. To prove G4 formation in the *hTERT* promoter G-rich sequence in the double-stranded supercoiling DNA, plasmid constructs based on the pRFPCER plasmid were obtained. The plasmids contained genes of fluorescent proteins (RFP and Cerulean) and sequence of the central G4 in the *hTERT* promoter region. G4 formation in the central *hTERT* promoter region in the obtained constructs was demonstrated with the DNA polymerase stop assay. The influence of G228A and G250A substitutions on G4 stability under physiological conditions was investigated. It was established that the low-molecular weight ligands BRACO19 and TMPyP4, the well-studied stabilizers of the G4 structure, can effectively interact with the *hTERT* promotor central G4 in the range of concentrations 5-25 μM.

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Keywords: *hTERT* promoter, human telomerase reverse transcriptase, G-quadruplex, G4-stabilizing ligands, “driver” mutations

INTRODUCTION

Telomerase comprises a ribonucleoprotein complex responsible for maintenance of the length of telomers, and it is considered vitally important for the cell. Human telomerase reverse transcriptase (hTERT) is usually inactive in somatic cells (with stem cells being an exception), and its overexpression is observed in 85-90% of malignant tumors [1]. Under normal conditions, *hTERT* gene expression is controlled by the transcription factors SP1, c-Myc, BRCA1/2. In the case of driver mutations G228A or G250A (positions1295228 and 1295250 of the human chromosome 5, respectively) additional binding sites for the ETS (Erythroblast Transformation Specific) or TCF (ternary complex factors) factors appear in the *hTERT* gene promoter region, which results in the *hTERT* expression increase [2, 3].

The *hTERT* promoter contains a GC-rich region with a length of 68 nucleotides. Presence of 12 tracts of three or more 2′-deoxyguanosine residues in the G-rich strand creates prerequisite for three tandems parallel G4 formation under certain *in vitro* conditions [4-6] (Fig. 1). It is considered that G4 structures formation in the promoter region prevents human DNA- and RNA-polymerases binding, and, likely, plays

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an important role in the *hTERT* gene expression regulation [4, 5]. According to one of the hypotheses, G228A or G250A substitutions appearance in the central G4 results in G4 structure destabilization, which makes this sequence more accessible for binding to various enzymes and transcription factors [2, 3]. Hence, G4-structure stabilization in the promoter could be considered as a possible way to reduce expression of the *hTERT* gene and tumor growth.

To investigate formation of G4 under physiological conditions plasmid constructs that contain a reporter gene (such as systems based on luciferase or GFP) and a sequence potentially capable of forming G4 are often used. Such constructs serve as convenient tools in investigation of G4 structures and their effects on gene expression, because they allow to examine structures of any topology and varying thermodynamic stability [7, 8]. Considering that G4-structures are of interest from the point of view of disease therapy, these reporter constructs could also be used as a tool for selecting most effective low-molecular weight ligands that affect G4 stability under physiological conditions.

A large number of G4 stabilizing ligands has been suggested so far. One of the best-investigated G4 ligands is BRACO19 (3,6,9-trisubstituted derivative of acridine – N,N′-(9-(4-(dimethylamine)phenylamino) acrydin-3,6-diyl)bis(3-(pyrrolidine-1-yl)propane amide) hydrochloride), which exhibits high affinity to telomeric G4 and is able to suppressing telomerase transcription [9]. TMPyP4 (5,10,15,20-tetrakis-(N-methyl-4-pyridyl)porphin) is also often used as a G4-stabilizing ligand capable of inhibiting telomerase activity [10]. However, the exact mechanism of its action is not known yet. PhenDC3 (3,3′-[1,10-phenantroline-2,9-diyl-bis(carbonylamino)]bis[1-methylquinoline] 1,1,1-trifluoromethane sulfonate) also can bind telomeric G-rich repeats and it is widely used *in vivo* for G4 detection [11, 12].

The goal of this study was development of reporter constructs containing genes of two fluorescent proteins (RFP and Cerulean) and a sequence of the central G4 in the promoter region of the wild type *hTERT* or of the gene with driver mutations; as well as analysis of the effect of G4 on reporter protein gene expression. In the course of the study, it was necessary to design the reporter construct and construct of the double strand sequences inserts required by the type of analysis, to evaluate the possibility of formation and thermodynamic stability of the investigated G4 using single-stand models, and to test the G4 formation possibility in the plasmid DNAs using polymerase stop assay. One other goal was investigation of the effects of G4-stabilizing ligands (BRACO19, TMPyP4, PhenDC3) on reporter gene expression in bacterial system in order to examine the possibility of the produced constructs application in the primary screening of G4-stabilizing compounds, which could be promising in treatment of various human diseases.

MATERIALS AND METHODS

Oligonucleotides and plasmid DNAs. Oligonucleotides and primers used in the study are presented in Table 1. Oligonucleotides were produced using a standard phosphoramidite technique and purified with electrophoresis in polyacrylamide gel (Evrogen, Russia). Prior to conducting experiments, oligonucleotides were additionally precipitated in ethanol

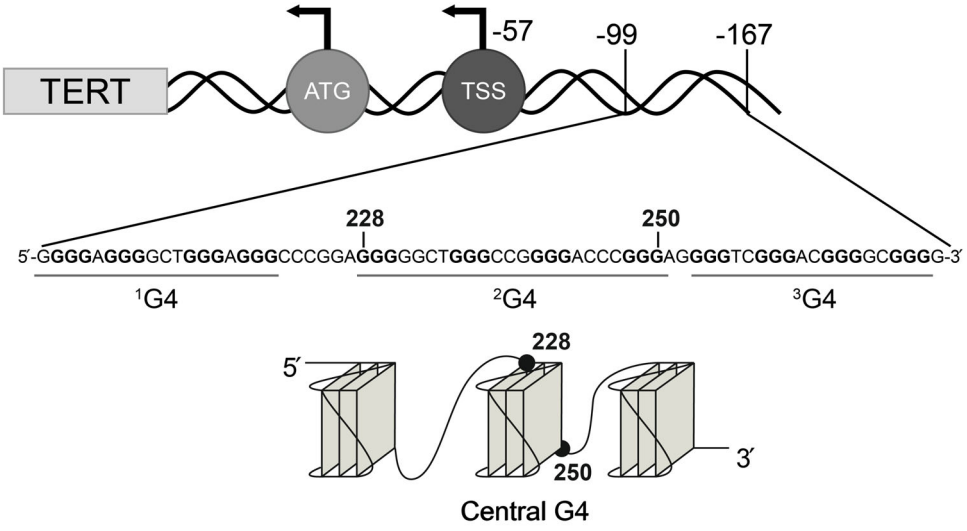


Fig. 1. Structural organization of the G-rich promoter region of the *hTERT* gene. Location of driver mutations in the central G4 at positions -124 and -146 from the start codon (ATG) (positions 1295228 and 1295250 of the human chromosome 5, respectively) are marked. TSS, transcription start site.

Table 1. Oligonucleotides used in the study

Name	Oligonucleotide sequence (5′→3′)
41-WT	pGGATCCCGGGTCCCCGGCCCAGCCCCCTCCAGGAGATATCA
41-C228T	pGGATCCCGGGTCCCCGGCCCAGCCCCTTCCAGGAGATATCA
41-C250T	pGGATTCCGGGTCCCCGGCCCAGCCCCCTCCAGGAGATATCA
41-C228T/C250T	pGGATTCCGGGTCCCCGGCCCAGCCCCTTCCAGGAGATATCA
41-c-Myc	pGGATCCTTCCCCACCCTCCCCACCCTCCCCAGGAGATATCA
45-WT	pTATGATATCTCC***TGGAGGGGGCTGGGCCGGGGACCCGGG***ATCCGC
45-G228A	pTATGATATCTCC***TGGAAGGGGGCTGGGCCGGGGACCCGGG***ATCCGC
45-G250A	pTATGATATCTCC***TGGAGGGGGCTGGGCCGGGGACCCGGA***ATCCGC
45-G228A/G250A	pTATGATATCTCC***TGGAAGGGGGCTGGGCCGGGGACCCGGA***ATCCGC
45-c-Myc	pTATGATATCTCC***TGGGGAGGGTGGGGAGGGTGGGGAAGG***ATCCGC
Stop_assay_primer	(TAMRA)-TGCAGGTCGACAAGCTTGG
Cer_fw	TGAGCAAGGGCGAGGAGC
Cer_rev	TGGTGCAGATGAACTTCAGG
RFP_fw	GCTGATCAAGGAGAACATGC
RFP_rev	AGGATGTCGAAGGCGAAGG

Note. G-rich sequences potentially capable of forming G4 are highlighted with semi-bold italic font. Positions with nucleotide substitutions are marked with gray.

according to the standard technique [13]. Concentration was determined with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). Molar extinction coefficients for DNA oligonucleotides were calculated with the help of on-line service OligoAnalyzerTM Tool (https://www.idtdna.com/calc/analyzer).

A pRFPCER plasmid with a size of 3796 base pairs (bp) was provided by I. A. Osterman (Faculty of Chemistry, Lomonosov Moscow State University). It has been successfully used for analysis of low-molecular antibacterial compounds action mechanisms [14-16]. In the initial pRFPCER ampicillin resistance gene was replaced with the kanamycin resistance genes using the standard protocol and a NEBuilder® HiFi DNA Assembly kit (NEB, United Kingdom).

Determination of the formation possibility and thermodynamic stability of G4 using circular dichroism (CD) method. Samples (1 ml) of oligonucleotides 45-WT, 45-G228A, 45-G250A, 45-G228A/G250A, 45-c-Myc with absorption 0.5-0.6 were incubated at 95°C for 15 min in an 8 mM potassium phosphate buffer (5.7 mM K_2HPO_4; 2.2 mM KH_2PO_4; pH 7.1) containing 100 mM KCl followed by graduate cooling for 16-18 h to room temperature. Circular dichroism spectra were recorded in a quartz cuvette (Hellma Analytics, Germany) with optical path length of 1 cm in a temperature range from 20 to 85°C with 5°C-step at average heating rate 1°C/min with a Chirascan spectrophotometer (Applied Photophysics Ltd., United Kingdom). Spectra were recorded in the range 220-340 nm at scanning rate 30 nm/min and time of signal averaging of 2 s under conditions of continous flow of dry nitrogen. The obtained spectra were smoothed with the Savitzky–Golay filter. Melting temperature (T_m) was defined as a temperature at which 50% of the sample was denatured. Errors in T_m determination were calculated based on the standard deviations of the thermoregulator temperature [17].

Preparation of plasmid constructs containing GC-rich inserts. A plasmid vector pRFPCER (200 ng) was hydrolyzed in 50 µl of reaction medium including 5 µl of 10× buffer rCutSmart™ for restriction (New England BioLabs, USA) and restriction endonuclease (RE) SacII (10 units/µl, 5 µl) and RE NdeI (10 units/µl, 5 µl) (Thermo Fisher Scientific) for 3 h at 37°C. Reaction product was purified in a 1% agarose gel and isolated from the gel with phenol-chloroform extraction [18].

DNA duplex insert was formed from synthetic 5′-phosphorylated oligonucleotides with length 41 and 45 nucleotide residues (nt) (Table 1). For this purpose, a mixture of 41- and 45-mer oligonucleotides with concentration 0.4 μM in water was incubated for 10 min at 95°C followed by gradual cooling to room temperature for 3 h. The otained DNA duplex had sticky ends required for insertion into a linearized vector. Next, 50 ng of a linearized vector and 100-fold excess of DNA duplexes were ligated with T4 DNA-ligase (5 units; Thermo Fisher Scientific) in a corresponding buffer solution (Thermo Fisher Scientific) and incubated for 1 h at 37°C. After ligation the mixture (5 μl) was used for transformation of *Escherichia coli* XL1-Blue cells using heat-shock method [19].

Plasmid DNA was isolated from cell colonies with a MiniPrep BC021S kit for DNA isolation (Evrogen). Accuracy of the construct sequence was confirmed by sequencing in the Center for Collective Use 'Genom' (Russia). *E. coli* JW5503 cells were transformed with the obtained plasmid constructs.

Analysis of G4 formation in the plasmid constructs using polymerase stop assay. A PCR-mix (10 μl) was prepared for each construct containing 1 μl of 10× buffer for *Taq*-polymerase with 50 mM KCl (Thermo Fisher Scientific), 0.2 μl of 10 mM solution of deoxynucleotide triphosphates, 4 mM $MgCl_2$, *Taq*-polymerase (0.1 μl; final activity – 0.5 units; Thermo Fisher Scientific), 1 μl of 10 μM Stop_assay_ primer solution, and 50 ng of plasmid DNA solution. KCl concentration was increased to 100 mM to facilitate G4 formation. PCR conditions: initial denaturation 3 min at 95°C followed by 35 cycles (denaturation 30 s at 90°C, primer annealing 1 min at 53°C, extension 1 min at 72°C); final extension 5 min at 72°C. Reaction products were separated using electrophoresis in 10% polyacrylamide gel with 7 M urea. A standard 1× TBE-buffer was used for electrophoresis. Visualization of fluorescent regions was carried out with a Typhoon FLA 9500 imager (GE Healthcare Bio-Sciences AB, Japan).

Determination of the reporter gene transcription efficiency in *E. coli* JW5503 cells containing plasmid constructs. Cellular RNA was isolated with an ExtractRNA solution (Evrogen) according to the manufacturer's instructions. Concentration of obtained RNAs was determined with a spectrophotometer.

An aliquot (1 μl) from each sample was treated in 20 μl of a reaction mixture with addition of 2 μl of 10× buffer for DNAse and 1 μl of DNAse I (Thermo Fisher Scientific) at 37°C for 10 min. Next, reverse transcription reaction was carried out. Reaction mixture (20 μl) contained 8 μl of 2.5× mixture for reverse transcription (Sintol, Russia), 1 μl of random hexamer primer mix, 2 μl of 20 mM DTT, 1.5 μl of MMLV revertase solution (Moloney murine leukemia virus reverse transcriptase) (50 U/μl; Sintol), 0.5 μl of Ribolock RNAse inhibitor (40 U/μl; Thermo Fisher Scientific), and 7 μl of solution of RNA treated with DNAse. PCR mix (20 μl) contained 4 μl of 5× qPCRmix-HS SYBR (Evrogen), 0.8 μl of 10 μM mixture of the primers to the gene encoding either Cerulean protein or RFP (Cer_fw/Cer_rev or RFP_fw/RFP_rev, respectively), 13.2 μl of water, and 2 μl of cDNA. PCR conditions: initial denaturation 5 min at 95°C followed by 40 cycles (denaturation for 30 s at 95°C, primer annealing for 30 s at 60°C, extension 30 s at 72°C). For each ligand experiment was conducted in two biological replicates, within one biological replicate four technical replicates were performed. PCR data processing was carried out with Excel software package. Significance of differences between the amount of mRNA in the cells with genetic constructs was evaluated with ANOVA [20].

Analysis of reporter proteins expression in *E. coli* JW5503 cells containing reporter constructs depending on concentration of G4 stabilizing ligands. Overnight cultures of *E. coli* JW5503 cells containing reporter constructs pContr, pWT, pG228A, pG250A, pG228A/G250A, or pc-Myc, were diluted to optical density of ~0.01 at 600 nm. G4-Stabilizing ligands BRACO19, TMPyP4, PhenDC3 (Sigma-Aldrich, USA) were dissolved in DMSO to concentrations 200, 400, 1000 μM and 5-μl aliquots were placed into wells of 96-well plates (Medpolimer, Russia). Volume in each well was adjusted to 200 μl with diluted cull culture. Microplates with cells were incubated for 24 h at 37°C and shaking at 220 rpm. Next, plates with cells were centrifuged at 4°C (4200 rpm; Eppendorf, Germany), washed with 0.9% NaCl solution followed by measuring optical density at 600 nm and fluorescence of proteins Cerulean (excitation/emission wavelengths 430/491 nm) and RFP (545/595 nm) with a Synergy H1 plate reader (BioTek Instruments (Agilent), USA). To evaluate relative fluorescence of the Cerulean protein, fluorescence intensity of Cerulean was divided by the intensity of fluorescence of RFP. Fluorescence of 0.9% NaCl solution was not taken into consideration in calculations, because it was significantly lower than the standard deviation for the protein fluorescence. For each ligand experiment was carried out in two biological replicates, and within one biological replicate four technical replicates were performed.

RESULTS

To confirm G4 formation in the *hTERT* gene promoter region in supercoiled genomic DNA in the cells and to determine effect of the G4 stability on the reporter protein gene expression, reporter systems were developed containing fragment of the *hTERT* gene

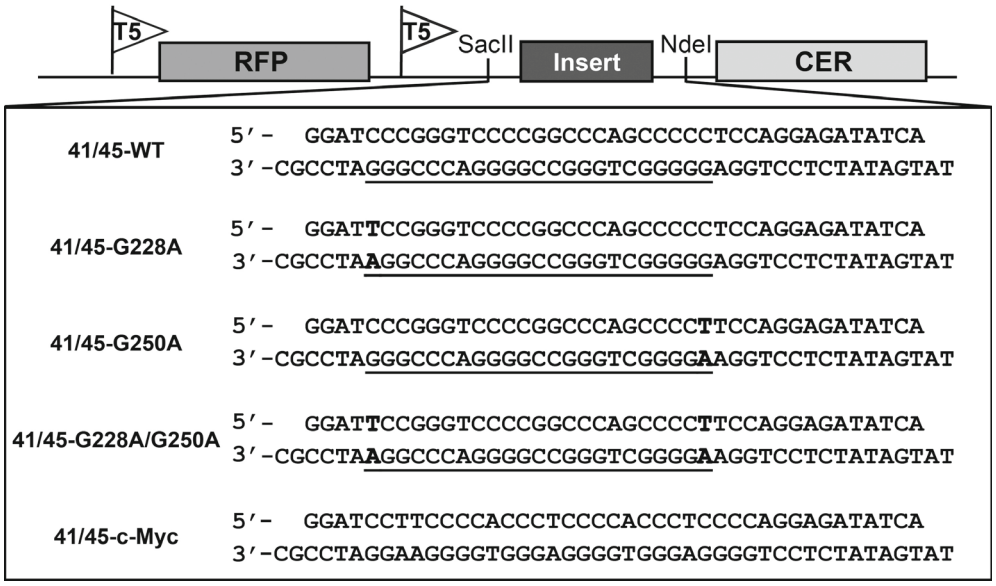


Fig. 2. Schematic representation of the reporter construct used for G4 structures detection. The inserted fragment contained the *hTERT* gene promoter region central G4 (underlined); positions 228 and 250 are highlighted with semi-bold font.

promoter region as an insert to the 5′-untranslated region (UTR) of the reporter gene (Fig. 2). Advantage of using prokaryotic systems in the case of the *hTERT* gene promoter region is in the fact of exclusion of the effects of the human transcription factors on the protein synthesis.

The pRFPCER plasmid DNA containing genes of two fluorescent proteins, RFP and Cerulean, was used as a basis [14, 15]. In this construct, RFP and Cerulean transcription is under control of two identical, but individual T5 promoters (Fig. 2). Hence, one of the fluorescent proteins, RFP, plays a role of internal control, and facilitates monitoring of the certain process by eliminating effects of other intracellular factors; while the second protein, Cerulean, facilitates monitoring of G4 formation. The ratio of the Cerulian fluorescence to RFP fluorescence served as an analytical signal.

Two issues were considered during design of the reporter constructs. 1) Considering that the G-rich region of the *hTERT*-promoter usually forms a multicomponent G4 structure, it would be extremely difficult to differentiate the effect of a single nucleotide substitution on the particular G4. 2) Insertion of the long G-rich region of the *hTERT*-promoter to the plasmid DNA could change significantly the protein transcription level independent on G4 formation. In addition, we attempted to minimize the length of the insert, in order to eliminate any effects on mRNA stability and transcription factors recognition sites formation. Based on these considerations, it was decided to use the 41/45-mer insert containing fragment of the *hTERT* promoter region only with the central G4 with native structure and with substitutions G228A and G250A. Despite the fact that the double substitution G228A/G250A has not been observed in nature, we also used oligonucleotide with both substitutions, because we expected more significant destabilization of the G4 structure in this case. The G4 sequence from the *c-Myc* promoter that had been investigated in detail both *in vitro* and *in vivo* [21, 22] was used as a reference.

It is believed that location of the G4 structure in the 5′-UTR of the gene decreases the transcription level and, hence, the protein synthesis level [7]. We hypothesized that the synthesis of Cerulean protein would be dependent on stability of the G4 formed in the G-rich insert comprising the *hTERT*-promoter fragment. In other words, the more stable is G4 in front of the Cerulean gene, the lower is the synthesis of the protein and, correspondingly, the lower Cerulean fluorescence signal is observed.

Before the production of reporter constructs, it was necessary to confirm that G4 indeed could be formed in the 45-mer oligonucleotides. The used oligonucleotides comprised non-modified sequences, and contained substitutions at positions 228 and 250 (G>A). To solve this problem, we used circular dichroism method.

Secondary structures that differ from the canonical B-form of DNA, such as G4 and hairpins, could be formed also in the plasmids with negative supercoiling [23, 24]. G4-structures are considered less stable thermodynamically and more labile in comparison with double helix. That is why it was important to demonstrate that G4 indeed could be present in the context of double helix in these plasmids. To confirm G4 formation in the plasmid constructs, polymerase stop assay was used.

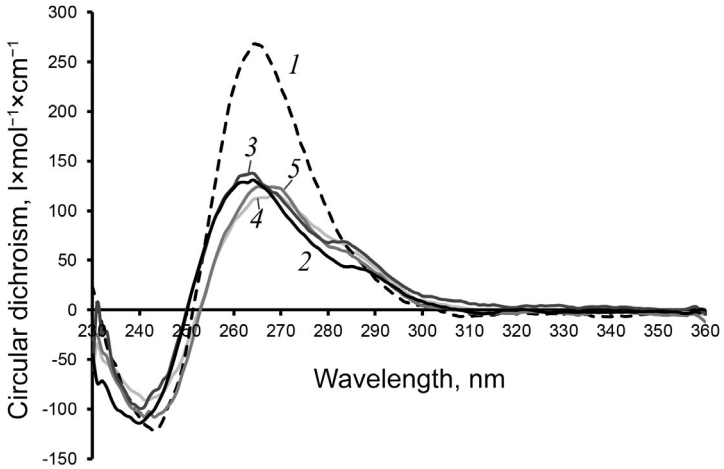


Fig. 3. CD spectra of the 45-mer oligonucleotides. Curves: *1*) 45-c-Myc, *2*) 45-WT, *3*) 45-G250A, *4*) 45-G228A, *5*) 45-G228A/G250A.

In our plasmid constructs the G4 of the promoter region of the *hTERT* gene is located in the sense strand, hence, effect of the G4 structure on transcription of the reporter *Cerulean* gene could be expected. Effect of G4 stability on the mRNA synthesis of the Cerulean protein was demonstrated using reverse-transcription quantitative PCR.

In order to evaluate the possibility of using G4-stabilizing ligands for reducing efficiency of the *hTERT* expression in the case of presence of driver mutations, fluorescent properties of the cells with produced reporter constructs in the presence of BRACO19, TMPyP4, and PhenDC3 were investigated.

Design of the reporter constructs. Use of DNA duplexes with length 41/45 bp containing the *hTERT* gene promoter region central G4 sequences with G228A, G250A substitutions and without them were suggested as fragments of the 5′-UTR of the reporter gene (Fig. 2). In addition to the *hTERT* gene promoter region the DNA duplexes contained the Shine-Dalgarno sequence, which is cut out from the DNA of the pRFPCER plasmid by the REs SacII and NdeI. The initial plasmid pRFPCER (pContr) was used in the experiments as a control to evaluate effect of introduction of the 41/45-mer insert on the process of Cerulean protein synthesis.

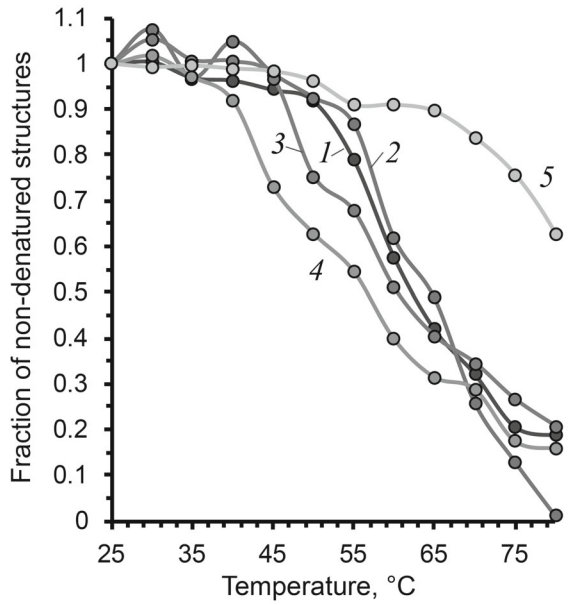
Prior to reporter constructs production, it was necessary to confirm that G4 could indeed be formed in the 45-mer oligonucleotides. It was shown that the *hTERT* gene promoter region central G4 could be formed already in the 30-mer models [25]. However, the oligonucleotides used in our study contained flanking regions, which could affect negatively the G4 stability.

Determination of G4 thermodynamic stability in 45-mer single-strand models. To evaluate the possibility of G4 structure formation and effect of G228A and G250A substitutions on the G4 stability with CD spectroscopy, the 45-mer models of single-strand DNAs containing sequence of the *hTERT* gene promoter region central G4 were investigated (Table 1). The obtained CD spectra of 45-WT, 45-G228A, 45-G250A, 45-G228A/G250A, and 45-c-Myc have characteristic maximum at 265 nm and minimum at 245 nm, which indicate formation of a parallel G4 (Fig. 3). In general, the values of circular dichroism of the 45-c-Myc are 1.5-2.0-fold higher than for oligonucleotides of *hTERT* promoter region G4.

Melting curves for the structures are presented in Fig. 4a from which their T_m were calculated (Fig. 4b). As expected, the G4 structure of 45-WT exhibits higher T_m, than the G4 formed from oligonucleotides with G-A substitutions. Presence of one substitution, either G228A or G250A, only insignificantly affects stability of the G4 structure, T_m differ by 1-2°C. At the same time, T_m of the G4 with two substitutions 45-G228A/G250A differs from the T_m of the 45-WT by 8°C, this means that introduction of the second nucleotide substitution results in additional destabilization of G4. It was not possible to evaluate accurately T_m of the G4-structure formed from 45-c-Myc, since this structure remained stable at 80°C. Hence, the 45-mer G-rich oligonucleotides 45-WT, 45-G228A, 45-G250A, 45-G228A/G250A, 45-c-Myc form sufficiently stable G4 in the presence of 100 mM KCl under the used experimental conditions at 37°C.

Analysis of G4 formation in plasmid constructs. To confirm formation of G4 in the plasmid constructs the polymerase stop assay was used. The method is based on the fact that G4 is an obstacle for *Taq*-polymerase, which inhibits the DNA strand elongation on the template during the enzymatic reaction. As a result, a shorter product is formed, which could be detected using electrophoresis in polyacrylamide gel. In order to increase stability of G4 under conditions of PCR, KCl was added to the reaction mixture

a



b

Oligonucleotide	T_m ± 1, °C
45-WT	65
45-G228A	64
45-G250A	63
45-G228A/G250A	57
45-c-Myc	>80

Fig. 4. Melting curves obtained using CD spectroscopy at 265 nm (a); T_m of G4-structures formed from the used oligonucleotides (b). Designations on the panel (a), curves: *1*) 45-WT, *2*) 45-G228A, *3*) 45-G250A, *4*) 45-G228A/G250A, *5*) 45-c-Myc.

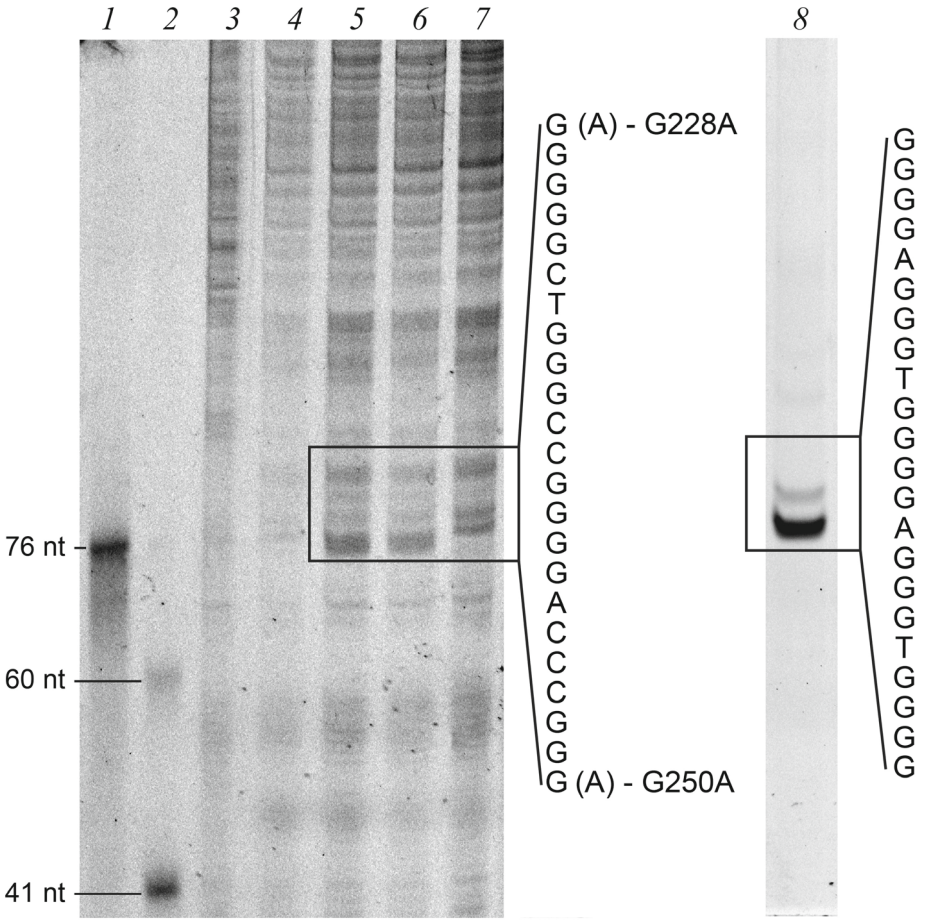


Fig. 5. Products of the DNA strand elongation by *Taq*-polymerase. pContr, pG228A/G250A, pWT, pG228A, pG250A, pc-Myc were used as templates in the presence of KCl (lanes *3*-*8*, respectively). Lanes *1* and *2*) markers with length 76 nt and 60 + 41 nt, respectively.

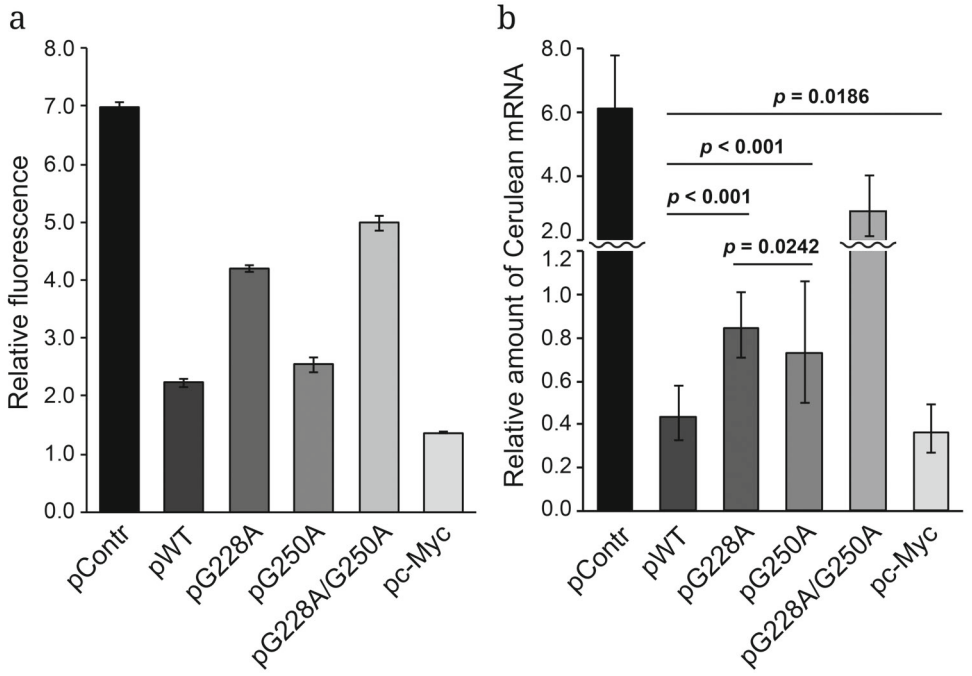


Fig. 6. Ratio of fluorescence of the Cerulean/RFP proteins in the *E. coli* cells containing constructs obtained in this study (a); transcription efficiency of the *Cerulean* gene in these constructs (b). To evaluate significance of differences between the amounts of mRNA in the cells with different genetic constructs ANOVA (one-way analysis of variance) was used. *p*-values are shown.

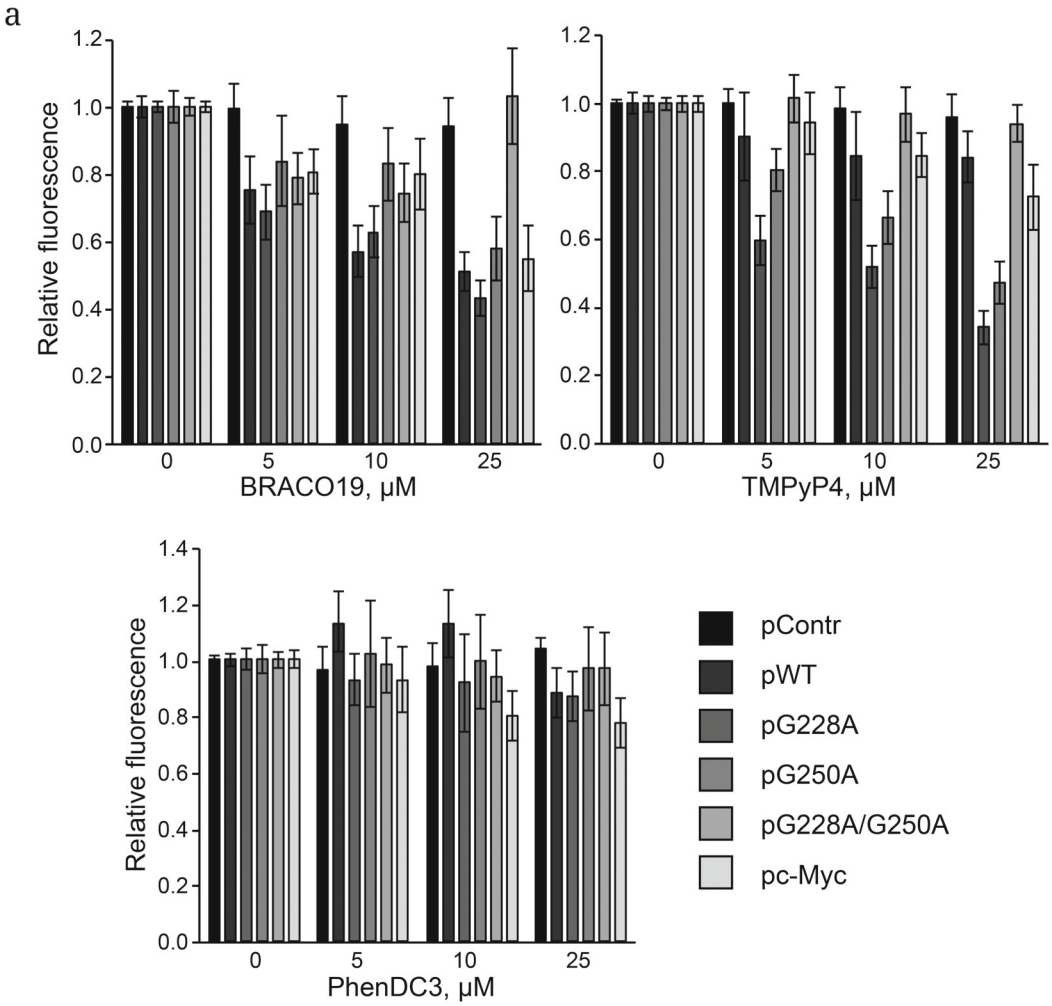
up to concentration 100 mM. A product with length around 76 nt was expected to be formed. It can be seen in Fig. 5 that the 'stop' of *Taq*-polymerase occurs in the plasmids pWT, pG228A, pG250A, and pc-Myc.

Evaluation of reporter gene expression in the *E. coli* cells with plasmid constructs containing sequence of the *hTERT* gene promoter region central G4. Analysis of the reporter constructs with two fluorescent proteins in the *E. coli* JW5503 cells demonstrated significant difference in the fluorescence signal between the cells transformed with pContr, pWT, pG228A, pG250A, pG228A/G250A, and pc-Myc (Fig. 6a). The pContr plasmid was used as a control for reporter protein fluorescence, the pc-Myc construct was used as a reporter gene repression efficiency control due to formation of G4, because this construct contains insert of the same length as the *hTERT* promoter region G4, but it forms a more stable G4 in comparison with the former (Fig. 4b). In can be seen in Fig. 6 that the cells with pContr and pG228A/G250A exhibited higher ratio between the fluorescent signals of Cerulean and RFP, and the cells with pWT and pc-Myc exhibited the lowest ratio. In the case of the cells with pWT plasmid the 3.2-fold decrease of the relative fluorescent signal of the Cerulean protein was observed in comparison with the pContr and the 1.6-fold increase in comparison with the pc-Myc. The decrease of the Cerulean protein fluorescence in the case of the pG250A plasmid was comparable with the case of pWT. Regarding of pG228A and pG228A/G250A the fluorescence signal of Cerulean protein differed from the instance of pContr approximately by 1.7- and 1.4-fold, respectively.

Analysis of the effect of G4 on reporter gene mRNA synthesis. Effect of the G4 stability on the mRNA synthesis was demonstrated using reverse-transcription quantitative PCR. It can be seen in Fig. 6b that the amount of mRNA for each construct correlates with the Cerulean/RFP relative fluorescence level. The highest amount of mRNA was observed in the case of the cells with the pContr construct, the amount of mRNA in the cells with the pG228A/G250A construct was almost 2-fold lower. In all other cases the amount of mRNA was significantly lower: ~6-12-fold lower in comparison with the mRNA from pContr and ~3-6-fold lower in comparison with pG228A/G250A. The mRNA levels in the cells with pWT, pG228A, pG250A, pc-Myc differ not so much, but the differences are statistically significant (significance level $\alpha = 0.05$).

Effect of low-molecular weight ligands stabilizing G-quadruplexes on synthesis of reporter protein. Fluorescent properties of the cells with the obtained reporter constructs were investigated in the presence of G4-stabilizing ligands. For this purpose, the most popular and well-characterized G4-stabilizing ligands were used: BRACO19, TMPyP4, PhenDC3 [26-28]. The histograms presented in Fig. 7 show relative fluorescence signal of the Cerulean/RFP proteins in the presence of different ligand concentrations normalized to the signal observed without addition of any ligand.

a



b

Ligand, μM \ Plasmid		pContr	pWT	pG228A	pG250A	pG228A/ G250A	pc-Myc
BRACO19	0.0	1.00 ± 0.01	1.00 ± 0.03	1.00 ± 0.01	1.00 ± 0.05	1.00 ± 0.02	1.00 ± 0.01
	5.0	1.0 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
	10.0	0.95 ± 0.08	0.6 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.8 ± 0.1
	25.0	0.9 ± 0.1	0.5 ± 0.1	0.43 ± 0.05	0.6 ± 0.1	1.0 ± 0.1	0.55 ± 0.10
TMPyP4	0.0	1.00 ± 0.01	1.00 ± 0.03	1.00 ± 0.02	1.00 ± 0.02	1.00 ± 0.02	1.00 ± 0.02
	5.0	1.00 ± 0.04	0.9 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	0.9 ± 0.1
	10.0	1.0 ± 0.1	0.85 ± 0.13	0.5 ± 0.1	0.7 ± 0.1	1.0 ± 0.1	0.85 ± 0.06
	25.0	1.0 ± 0.1	0.8 ± 0.1	0.34 ± 0.05	0.5 ± 0.1	0.9 ± 0.1	0.7 ± 0.1
PhenDC3	0.0	1.00 ± 0.01	1.00 ± 0.02	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.03	1.00 ± 0.03
	5.0	1.0 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	1.0 ± 0.2	1.0 ± 0.1	0.9 ± 0.1
	10.0	1.0 ± 0.1	1.1 ± 0.1	0.9 ± 0.2	1.0 ± 0.2	0.9 ± 0.1	0.8 ± 0.1
	25.0	1.04 ± 0.04	0.9 ± 0.1	0.9 ± 0.1	0.97 ± 0.15	1.0 ± 0.1	0.8 ± 0.1

Fig. 7. Histograms of dependence of Cerulean/RFP relative fluorescence in the cells with different reporter constructs (a) normalized to fluorescence of the cells without ligand present on the concentration of the BRACO19, TMPyP4, PhenDC3 ligands in the concentration range 0-25 μM. Number of measurements: n = 6, confidence level: P = 0.95. Accurate values of the normalized relative fluorescence of the Cerulean/RFP proteins are shown (b).

Using of relatively low concentration of BRACO19 (5 μM) results in significant (20%) decrease of the Cerulean expression in the cases of pWT, pG228A, pG250A, and pc-Myc. No significant differences between the signals were observed in the calls with pContr and pG228A/G250A. Using BRACO19 at concentration 25 μM resulted in almost 2-fold decrease of the Cerulean fluorescence in the cells with all used constructs, except pContr and pG228A/G250A.

Addition of TMPyP4 did not decrease expression of the Cerulean protein in the case of pContr and pG228A/G250A. In the instance of pWT and pc-Myc constructs, addition of even 25 μM resulted only in insignificant decrease of the signal. At the same time, synthesis efficiency of the Cerulean protein in the cells with the constructs pG228A and pG250A decreased significantly at concentration of 5 μM, and at 25 μM it decreased more than 2-fold.

Addition of PhenDC3 does not result in noticeable changes of the fluorescence signal in the cells. Decrease of the Cerulean protein synthesis by 20% is observed in the cells with the pc-Myc construct at concentrations 10-25 μM.

DISCUSSION

G-quadruplex (G4) is an element of secondary structure in nucleic acids formed from the sequences enriched with 2′-deoxyguanosine residues. G4s could form structures of different topologies differing in directions of the strands and length and composition of the loops [29]. Numerous evidence reported in the literature indicates a key role of G4s in important biological processes, in humans in particular [30]. G4-Motifs were found in human protooncogenes including the best-known *c-Myc* [31], *VEGF* [32], *c-kit* [33], and *BCL2* [34].

Stabilization or destabilization of G4 structures could affect functioning of the G4-binding transcription factors, polymerases, and other enzymes, and, as a consequence, affect efficiency of the gene transcription [35]. Hence, development of therapeutic agents facilitating formation or unfolding of the specific G4 structures could be a promising direction for development of novel types of anti-tumor preparations. At present, ligands have been identified that are capable of specific binding to the G4 structure of particular topology, however, the attempts to bind the G4 structure in the vicinity of a particular gene so far failed. To improve selectivity of G4 ligands action, use of their conjugates with oligodeoxyribonucleotides ensuring binding of the ligand to the G4 structure only at the genome sites capable of complementary interactions with the guide DNA has been suggested [36]. Formation of G4 structure is potentially possible in the *hTERT* gene promotor region. This region could contain nucleotide substitutions found in tumor cells (G228A and G250A) [37, 38].

To test the hypothesis that *hTERT* overexpression could be due to G4 destabilization, we developed a reporter system containing the fragment of the *hTERT* gene promotor region that forms the central G4, as the 5′-UTR reporter gene. The system was based on the pRFPCER plasmid DNA containing genes of two fluorescent proteins, RFP and Cerulean. The similar constructs were successfully used for assessment of the effect of the loop length in G4 located in the reporter proteins promoter regions on transcription and translation in *E. coli* [8]. Previously, the luciferase system [39-41] and the system based on GFP [42] were successfully used for evaluation of the effects of nucleotide substitutions on the synthesis of the hTERT protein. The luciferase system was also used for evaluation of the effect of G4-stabilizing ligands on transcription of *c-Myc* [43], *BCL-2* [44], *c-kit* [45], and telomerase activity [46]. However, it should be noted that luciferase system demonstrates indirect response via oxidation of luciferin. The pRFPCER reporter system suggested in this study does not have this drawback: it provides direct response of the fluorescent proteins, which makes this system faster and easier to use.

To evaluate the possibility of formation of G4 structures and effect of G228A and G250A substitutions on G4 stability, CD spectroscopy was used. The obtained CD spectra for the 45-WT indicated formation of the parallel G4 (Fig. 3). It was shown that the G-rich 45-mer oligonucleotides (Table 1) 45-WT, 45-G228A, 45-G250A, 45-G228A/G250A, and 45-c-Myc form relatively stable G4 in the presence of 100 mM KCl under common experimental conditions (at 37°C).

The melting curves obtained with CD spectroscopy with detection at 265 nm demonstrated that the G4 structure formed from 45-WT has a higher T_m than the G4 structures formed from the sequences with single substitutions, and introduction of the second substitution resulted in the further destabilization of the G4 structure. It was important to show that G4s indeed could be present in the double helix context in the obtained plasmids. To confirm the G4 formation in the plasmid structure, the polymerase stop assay was used. The polymerase ‘stop’ in the pc-Myc plasmid was found to be more significant in comparison with the plasmids containing sequence of the *hTERT* gene promotor region central G4 (Fig. 5). Such a significant ‘stop’ was expected for the pc-Myc based on the ‘melting’ data in the single-strand model.

Two ‘stops’ occur on the pWT and pG228A plasmid DNA: one before the G4 structure providing the 76-nt product and on the loop, providing the longer products. Interestingly, in the plasmid with G250A substitution there is a third ‘stop’ of *Taq*-polymerase.

Testing of the 96-mer single-stranded oligonucleotides containing all three G4s of the *hTERT* promoter region using dimethyl sulfate probing method in our research group revealed that the G250A substitution causes destabilization not only in this G-tract, but also in the next one [47]. The same, likely, occurs in the plasmid. The double substitution G228A/G250A in the case of G4 from the *hTERT* gene promoter results in complete destruction of G4. As one could expect, no polymerase 'stop' was observed in the pG228A/G250A construct, same as in the case of pContr. Hence, the central G4 of the *hTERT* gene promoter region can be formed in the plasmid construct in the double helix context and under conditions of supercoiling. Despite the fact that the extension temperature in PCR (72°C) is higher than T_m of the structures in the single-strand models 45-WT, 45-G228A, 45-G250A (Fig. 4b), supercoiling of the plasmid DNA makes existence of G4 in the plasmid DNA possible.

Two bands in the gel were also observed in the case of pc-Myc. Considering that G4 from the *c-Myc* promoter contains 5 G-tetrads, it potentially could form structures with different topology [22, 48]. Hence, it could be hypothesized that emergence of the band with weaker intensity is associated with formation of the structure, in which the first G-tetrad does not participate [49].

Analysis of relative Cerulean/RFP fluorescence of the *E. coli* cells transformed with the constructs obtained in this study demonstrated that the change in fluorescence correlates with the ability of inserted sequences to form G4: the more stable is G4, the lower is the Cerulean fluorescence. Relative increase of the Cerulean/RFP fluorescence signal could be explained by the destruction (or absence) of the G4 structure in the cell, which interferes with the process of *Cerulean* gene transcription. Indeed, the highest relative fluorescence is observed for the cells containing constructs pContr and pG228A/G250A, and the lowest – for the cells with pWT and pc-Myc (Fig. 6a). It is known that introduction of G228A and G250A substitutions locally disrupt the G4 structure [47] and, consequently, should increase efficiency of the Cerulean protein synthesis in comparison with the wild type *hTERT* gene. It was found that the G228A substitution destabilizes G4 to a larger degree than the G250A substitution; and the double substitution significantly disrupts the G4 structure. As expected, the double substitution destabilizes the G4 structure more significantly than the single substitutions. Certain decrease of the relative fluorescence of the cells containing the pG228A/G250A construct in comparison with the pContr could be explained by both presence of a longer sequence before the *Cerulean* gene, and by partial formation of G4 at 37°C. Although destruction of this structure was shown under conditions of PCR, one should take into account that PCR occurs at high temperatures (extension at 72°C), which facilitates destruction of the *hTERT* gene promoter region G4 containing two substitutions, while effect of the steric factor associated with supercoiling seems to be insufficient to maintain the structure.

The method of reverse transcription quantitative PCR was used to demonstrate the effect of G4 stability on synthesis of the Cerulean protein mRNA. It was found out that the amount of mRNA for each construct correlates with the level of relative Cerulean/RFP fluorescence. The lowest amount of the *Cerulean* mRNA was synthesized in the cells with pWT and pc-Myc, which increased in the cells with pG250A and pG228A. Hence, the effect of G4 stability is observed at the level of transcription, which is manifested by the amount of mRNA of the *Cerulean* reporter gene.

With the goal to evaluate the possibility of using G4-stabilizing ligands for decreasing *hTERT* expression efficiency in the case of appearance of driver mutations, fluorescent properties of the cells containing the obtained reporter constructs were investigated in the presence of BRACO19, TMPyP4, PhenDC3 (Fig. 7). The selected ligands differ in efficiency of binding to G4. Dissociation constant values obtained using fluoresence titration method [50] and surface plasmon resonance technique [51] for the complexes with parallel G4s are in the range for BRACO19: 6×10^{-8}–7×10^{-7} M, for TMPyP4: 2×10^{-7}–2×10^{-6} M, for PhenDC3: 2×10^{-11}–2×10^{-6} M. According to these data PhenDC3 exhibits the most pronounced ability to bind G4 in comparison with BRACO19 and TMPyP4, however, it causes only minor stabilization of the G4 from pc-Myc, the most stable among the G4s investigated in this study.

Based on our results, from the point of view of stabilization of the *hTERT* gene promoter region central G4, the best ligand is BRACO19 (decrease of expression of the Cerulean protein in the case of pWT, pG228A, pG250A, and pc-Myc by 20% at 5 μM concentration of BRACO19), because it is capable to produce a significant decrease of the Cerulean protein synthesis even in the cells containing constructs with stable G4 (pWT and pc-Myc). However, this compound is rather toxic, which limits its application [52].

In our experiments addition of TMPyP4 did not cause death of the *E. coli* cells containing reporter constructs at the ligand concentrations up to 80 μM (data not shown). Hence, unlike the BRACO19, TMPyP4 is not toxic for the cells, but its addition causes only the minor difference in the level of the Cerulean protein synthesis in the cells with the constructs containing stable G4 (~20% for the pWT and pc-Myc at 25 μM of TMPyP4). TMPyP4 preferably binds to the destabilized G4-structures, because the decrease in the Cerulean fluorescence in the cells containing constructs

pG228A and pG250A is much more pronounced. This observation is of fundamental importance, and sets the stage for the development of G4 ligands distinguishing stable and unstable structures.

Hence, the reporter system developed in this study could be used for evaluating stability of G4, including on addition of low-molecular ligands of various structures.

CONCLUSIONS

Reporter constructs were generated in this study that contain genes of two fluorescent proteins, RFP and Cerulean, and fragment of the sequence of the *hTERT* gene promotor region containing sequence of the central G4 of either the wild type or with driver mutations. Effect of the driver substitutions G228A and G250A on stability of G4 was investigated *in vitro* and under physiological conditions. It was shown that the 45-mer G-rich oligonucleotides 45-WT, 45-G228A, 45-G250A, 45-G228A/G250A, 45-c-Myc form relatively stable G4 *in vitro*. The most stable G4 were shown to exist in the context of double helix in the obtained plasmids and even under conditions of PCR (primer extension was carried out at 72°C). Effect of G4 stability on synthesis of mRNA of the *Cerulean* reporter gene was demonstrated. It was shown that the synthesis of mRNA of the *Cerulean* gene in the cells with stable G4 in the genetic construct is reduced. It was found that the amount of mRNA for each construct correlated with the level of relative Cerulean/RFP fluorescence.

It was established that the level of relative Cerulean/RFP fluorescence in the cells decreases with increase of stability of G4 in the insert. It was shown that the low-molecular weight ligands, BRACO19 and TMPyP4, are capable of stabilizing the central G4 from the *hTERT* gene promoter region in the concentration range 5-25 μM. This is manifested by the decrease of the relative Cerulean/RFP fluorescence. Among the considered G4-stabilizing ligands, the highest attention from the point of view of drug development should be paid to TMPyP4 due to its higher specificity towards destabilized quadruplex structure. Hence, the reporter system suggested in this study could be used for evaluation of G4 stability, including in the cases with addition of G4-ligands of various structures and primary screening of stabilizers in the bacterial system.

Abbreviations

CD	circular dichroism
BRACO19	N,N′-(9-(4-(dimethylamine)phenylamino) acrydin-3,6-diyl)bis(3-(pyrrolidine-1-yl) propane amide) hydrochloride
G4	G-quadruplex
hTERT	human telomerase reverse transcriptase
PhenDC3	3,3′-[1,10- phenanthroline-2,9-diylbis(carbonylamino)]bis[1-methylquinoline] 1,1,1-trifluoromethane sulfonate
RE	restriction endonuclease
T_m	melting temperature
TMPyP4	5,10,15,20-tetrakis-(N-methyl-4-pyridyl) porphin

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Contributions

E. A. Kubareva and M. E. Zvereva – concept and supervision of the study; Yu. V. Iakushkina, M. V. Monakhova, and A. M. Arutyunyan – conducting experiments; Yu. V. Iakushkina, M. V. Monakhova, E. A. Kubareva, M. E. Zvereva, and L. A. Nikiforova – discussion of the results of the study; Yu. V. Iakushkina and L. A. Nikiforova – writing of the manuscript; M. V. Monakhova and E. A. Kubareva – editing text of the paper.

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Ethics approval and consent to participate

This work does not contain any studies involving human and animal subjects.

Conflict of interest

The authors of this work declare that they have no conflicts of interest.

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