

Antiproliferative activity of G-quartet-containing oligonucleotides generated by a novel single-stranded DNA expression system

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Recently, a novel single-stranded DNA (ssDNA) expression system that can generate intracellularly ssDNA or oligodeoxynucleotide (ODN) molecules has been developed. Previous studies showed that this ssDNA expression system is capable of generating DNA enzyme ODNs and triplex-forming oligodeoxynucleotides (TFOs) in cells. In this study, we constructed an ssDNA expression vector that can generate a G-quartet-containing ODNs, GRO29A, in cells. Similar to synthetic ODNs, vector-generated GRO29As were shown to have significant antiproliferative activities in a number of cancer cell lines. These results further demonstrate the potential application of ssDNA expression system in gene target validation and drug development.

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DNA molecules containing runs of guanines (G) can form four-stranded structures referred to as G-quartets or G-quadruplexes.¹ This structural motif likely plays important biological roles *in vivo* since it has been found in the telomeric region of chromosomal DNA as well as in the transcriptional regulatory regions in several oncogenes.² Recently, Bates et al reported that a 29-mer G-quartet containing the oligonucleotide (ODN), GRO29A, has potent antiproliferative activity in a number of cancer cell lines.³ The activity of this ODN appears to correlate to its binding to a cellular protein, nucleolin.³ It was further demonstrated that GRO29A-mediated antiproliferative activity is a direct result of its inhibition of DNA replication.⁴

Historically, ODNs have been delivered extracellularly. Many different strategies have been attempted to improve the effectiveness of ODN delivery with varying degrees of success.⁵ Recently, a single-stranded DNA (ssDNA) expression system was developed to generate ODNs intracellularly.⁶ Using this ssDNA expression system, a number of ODN-based molecules have been generated intracellularly such as (1) DNA enzyme ODNs for down regulating *c-raf* gene expression,^{6,7} and (2) triplex-forming ODNs (TFOs) for inducing genomic recombination.⁸

In this study, we report an application of this novel ssDNA expression system for generating G-quartet-containing ODN, GRO29A. Our results indicate that

GRO29As generated by an ssDNA expression vector have antiproliferative activities in a number of cancer cell lines.

Materials and methods

Vector construction

Double-stranded ODNs coding for GRO29A were prepared by annealing two ODNs, 5'-29A(PAC/ECO), 5'-TAACCACCACCACCACAACCACCACCACCAAG-3' and 3'-29A(PAC/ECO), 5'-AATTCTTTGGTGGTGGTGGTTGTGGTGGTGGTGGTGGTAAAT-3'. The annealed double-stranded ODNs were then subcloned into *PacI* and *EcoRI* sites of an ssDNA expression vector, pssXE⁹ and the resulting vector was designated as pssXE(29A). Another vector expressing a control ODN, GRO15B,³ was similarly constructed and designated as pssXE(15B). The sequences of two ODNs coding for GRO15B were: 5'-15B(PAC/ECO), 5'-TAATTGGGGGGGGTGGG-3' and 3'-15B(PAC/ECO), 5'-AATTCCCACCCCCCA-ATTAAT-3'. All ODNs were purchased from Integrated DNA Technologies, Inc. (Coralville, IA).

Cell transfection and growth assay

A549, MCF7 and HeLa cells, obtained from the American Type Tissue Collection, were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 100 µg/ml penicillin G sodium and 100 µg/ml streptomycin sulfate (Invitrogen,

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Carlsbad, CA) and incubated at 37°C in a humidified 5% CO₂ incubator.

Cells were seeded in six-well plates at a density of 1×10^5 cells per well the day before transfection. Cells were transfected with either pssXE(29A) or pssXE(15B), or pssXE using transfection reagents, Lipofectamine Plus (Invitrogen, Carlsbad, CA) for A549 and MCF7 cells and SuperFect (Qiagen, Valencia, CA) for HeLa cells, according to the manufacturer's instructions. Plasmid DNA (1 µg) was used for both A549 and MCF7 cell transfection and 2 µg of plasmid DNA was used for HeLa cell transfection. Forty eight hours after the transfection, antiproliferative activities were assayed using the MTT kit (Roche Molecular Biochemicals, Indianapolis, IN) as directed by the manufacturer. The experiments were performed in triplicate.

Results and discussion

Antiproliferative activities of GRO29A generated intracellularly

The ssDNA expression vector, pssXE, was designed to contain: (1) a Mouse Moloney leukemia viral reverse transcriptase (MoMuLV RT) gene coding for a truncated but fully active RT;¹⁰ (2) a primer binding site (PBS) along with some flanking regions of the promoter that are essential for the reverse transcription initiation by MoMuLV RT;¹¹ (3) an ODN coding sequence (SOI); and (4) a stem-loop structure designed for the termination of the reverse transcription reaction (TS) (Fig 1). Once the vector is transfected into the cell, RT will be expressed and then the newly synthesized RT uses endogenous tRNA^{Pro} as a primer that binds to the PBS on the 3' end of the RNA transcript for ssDNA synthesis.¹² After the termination of reverse transcription reaction, ssDNA is released when the template mRNA is degraded either by endogenous RNase H or the RNase H activity of RT.¹⁰

G-quartet-containing ODN, GRO29A, has been shown to inhibit cell growth in a number of cancer cell lines.³ To investigate the possibility of generating functional GRO29A intracellularly using ssDNA expression system, we firstly subcloned the coding sequences for both GRO29A and GRO15B into the ssDNA expression

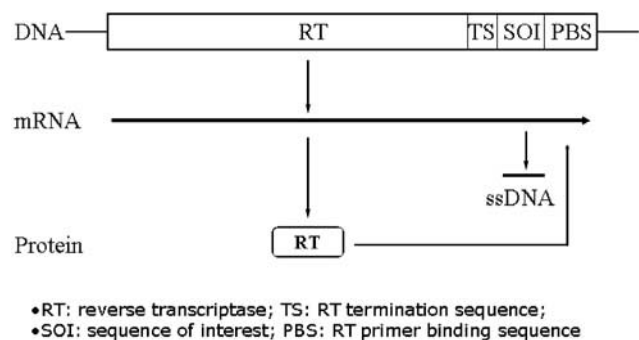


Figure 1 Design of the ssDNA expression system.

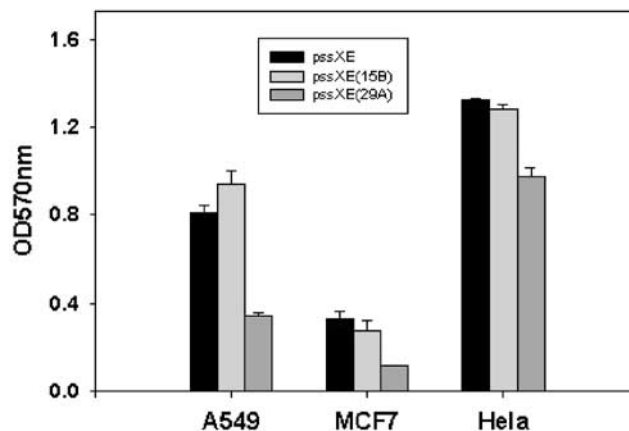


Figure 2 Antiproliferative activities of GRO29A generated by the ssDNA expression vector, pssXE in cancer cell lines. Cells were transfected with either pssXE(GRO29A) or pssXE(GRO15B). After treatment for 48 hours, viable cells were determined by the MTT assays. For details, see Materials and methods.

vector, pssXE. We then transfected these constructs into A549, MCF7, or HeLa cells and the antiproliferative activities were assayed using the MTT kit 48 hours after transfection. Figure 2 shows the results of MTT assays using the transfected cells. Compared to the cells transfected with control vector, pssXE(15B), various levels of inhibitory effects can be observed in all cancer cell lines transfected with pssXE(GRO29A) (A549 cells, 57.8%, MCF7 cells, 63.9%, HeLa cells, 26.4%). However, there were no significant differences between the cells treated with the control vector, pssXE (GRO15B) and the empty vector, pssXE.

Although various vectors have been designed to express ribozymes or antisense RNA molecules endogenously in target cells, the application of ODNs, mainly antisense ODNs, has been restricted to exogenous delivery.⁵ The ssDNA expression system we developed has been shown previously to generate ODNs like DNA enzymes and TFOs in cells.⁶⁻⁹ The results demonstrated in this study further indicate the potential application of the ssDNA expression system in gene target validation and drug development.

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