

ORIGINAL PAPERS

Antisense gene therapy using anti-k-ras and antitelomerase oligonucleotides in colorectal cancer

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ABSTRACT

Aim: to test the efficacy of anti-k-ras and antitelomerase oligonucleotides for disabling colorectal cancer cell growth.

Material and methods: an established human colorectal cancer cell line (SW 480, ATTC®) was used. Oligodeoxiribonucleotides (ODNs) have a phosphorotioate modification to ensure intracellular intake. We used an antitelomerase ODN (Telp5) and two anti-k-ras ODNs (AS-KRAS and ISIS). AS-KRAS is designed to join the k-ras oncogene's exon 1. ISIS links to the terminal transcription unit 5' of k-ras. Telp5 joins the template region of the hTR telomerase subunit. ODNs have been tested in different concentrations (1, 5, 10, 20 micromolar). Cell viability has been tested at 48 and 72 hours. Statistical analysis and graphic design were made with the statistical package "Analyzing Data with GraphPad Prism-1999", GraphPad Software Inc., San Diego CA®. We used the Student's t test for statistical analysis.

Results: the lowest dose (1 µM) was not effective. Using the highest dose (20 µM for 48 hours) of combined AS-KRAS and Telp5 cell viability decreased to 99.67%. The rest of results varied depending on ODN type, dose, and exposure time.

Conclusions: tested antisense ODNs stop colorectal cancer cell growth, and a combination of anti-telomerase and anti-k-ras is the most useful treatment. Efficacy is best with a higher dose and longer treatment period.

Key words: Colorectal cancer. Oligoribonucleotides. K-ras oncogene. Telomerase. Antisense therapy.

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INTRODUCTION

Colorectal cancer (CRC) survival after curative surgery depends on local and distant recurrence. After curative resection 5-year survival varies from 40 to 80% depending on local recurrence rates (1). With a 100% control of local disease, 17% of patients will die from cancer; with a local control of 80%, 31% of deaths will occur (2). These recurrences may result from tumoral cell spread during surgery (3,4).

The K-ras oncogene is activated in almost 70% of human CRCs (5). This is partly responsible for the uncontrolled growth of CRC cells (6). Its activation is also related to vascular endothelial growth factor (VEGF) hyperexpression, which is involved in angiogenesis (7).

DNA in telomeres shortens with each cellular division. In normal cells DNA shortens with each DNA replication, and therefore chromosomes become unstable, and cells cannot divide and eventually die.

Nevertheless, more than 90% of cancer cells have increased telomerase activity, which contributes to tumor cell uncontrolled growth (8). Telomerase activity is increased in 90-100% of human CRCs (9,10). This fact has been proposed as an alternative tumor development pathway (11).

Antisense techniques for gene therapy consist in blocking the expression of a gene using an oligonucleotide that is complementary to the gene sequence whose blocking is desired (12). Such an antisense nucleotide can be chemically designed, the simplest way to do so being the generation of a small piece of DNA: an oligodeoxiribonucleotide. Antisense sequences are designed in such a way that they recognize and link specific mRNA sequences. In this way, the double-chain hybrids generated cannot be translated by the ribosome into a protein, and protein translation cannot take place.

In our study antisense oligodeoxiribonucleotides (AS-ODN) have been designed to interfere with K-ras and

telomerase (hTERT subunit) genes. The aim of this study was to evaluate growth inhibition achieved *in vitro* in an established CRC cell line.

MATERIAL AND METHODS

An established human cell line was used: SW 480 (ATCC. *The Global Bioresource Center*[®]). This cell line comes from primary colorectal adenocarcinomas in stage B of Dukes' classification. It has a mutation in codon 273 of the p53 gene and a mutation in codon 12 of the protooncogene k-ras.

Cell viability studies have been made with the commercial kit Calcein-AM (Molecular Probes[®]). This procedure allows to identify living cells after incubation with modified calcein. Calcein-AM has a fluorescent activity that develops only when metabolized by viable cells.

AS-ODNs used have a phosphorotioate modification in order to prevent degradation by cellular nucleases, so intracellular intake is ensured. We designed an antitelomerase AS-ODN (Telp5) and two anti k-ras AS-ODNs (AS-KRAS and ISIS). The sequences of each AS-ODN are: ISIS 6957: 5'-CAG TGC CTG CGC CGC GCT CG-3'; AS-KRAS: 5'-AAG TTT ATA TTC AGT CAT-3'; Telp5: 5'-CCC TTC TCA GTT AGG GTT AG-3'.

ISIS 6957 has been previously used by other groups (13,14). The AS-KRAS tested in our study was designed to link exon 1 of the k-ras oncogene. ISIS joins the 5' terminal transcription unit of the k-ras oncogene. Telp5 links the telomerase hTR subunit. AS-ODNs were tested in different concentrations: 1, 5, 10 and 20 µM. We selected these amounts based on studies by other groups (13,15,16), but taking into consideration that we used no transfection vectors to facilitate cellular intake. No cytotoxicity has been described *in vitro* with concentrations

under 100 mM, nor toxicity in murine or ape models using intravenous doses under 100 mg/kg (12).

For viability/cytotoxicity experiments we used 96 cell culture plates in which treatments were applied in triplicate (three times).

After 24 and 72 hours of incubation with the various AS-ODN treatment combinations the effect on cellular viability was studied. For that purpose we used the "LIVE/DEAD viability/cytotoxicity kit" from *Molecular Probes*[®]. This kit contains modified calcein (calcein-AM), a substance with silenced fluorescent calcein. When this substance penetrates viable cells, calcein is metabolized and recovers fluorescence, which is then measured in relative fluorescence units (RFU).

For the statistical analysis and graphic design we used the program "Analyzing Data with GraphPad Prism-1999", GraphPad Software Inc., San Diego CA[®]. After determining the fluorescence (RFU) emission with each dose (1, 5, 10 and 20 µM) applied for 48 and 72 hours, a Student's t test was used to compare them with the RFU control group. A $p < 0.05$ was considered statistically significant. Although the statistical analysis was made using RFUs, our results are shown as rates of viability with respect to the control group, which presents a 100% viability rate, in order to facilitate reader comprehension (Table I).

RESULTS

Viability test with calcein-AM characterization

In order to know the optimal conditions for our study's technique, SW-480 cells were incubated with calcein for different periods of time, and the different fluorescent emissions were registered with a microplate cytofluorometer. Results (Fig. 1) show that a maximum of cellular fluo-

Table I. Statistical analysis: comparison of control group viability (RFU) vs. viability (RFU) after AS-ODN treatment

Control vs. ODN-AS 48 hours treatment	T Student (p)	Sig	Control vs. ODN-AS 72 hours treatment	T Student (p)	Sig
AS-KRAS 1 µM	0.0136	**	AS-KRAS 1 µM	1	NS
AS-KRAS 5 µM	0.005	**	AS-KRAS 5 µM	0.0002	***
AS-KRAS 10 µM	0.0005	***	AS-KRAS 10 µM	0.0002	***
AS-KRAS 20 µM	0.0003	***	-		
Telp5 1 µM	1	NS	Telp5 1 µM	1	NS
Telp5 5 µM	1	NS	Telp5 5 µM	0.0002	***
Telp5 10 µM	0.29	NS	Telp5 10 µM	0.0002	***
Telp5 20 µM	0.0007	***	-		
Anti K-T 1 µM	1	NS	Anti K-T 1 µM	1	NS
Anti K-T 5 µM	0.0006	***	Anti K-T 5 µM	0.0001	***
Anti K-T 10 µM	0.0004	***	Anti K-T 10 µM	< 0.0001	***
Anti K-T 20 µM	0.0001	***	-		
ISIS 1 µM	0.0513	NS	ISIS 1 µM	1	NS
ISIS 5 µM	0.0077	**	ISIS 5 µM	1	NS
ISIS 10 µM	0.0136	**	ISIS 10 µM	0.0011	**
ISIS 20 µM	0.0029	**	-		

Telp5 = antitelomerase; Anti K-T = AS-KRAS + Telp5; Sig = statistical significance; NS = no statistical significance. $p < 0.005 = *$; $p < 0.02 = **$; $p < 0.001 = ***$

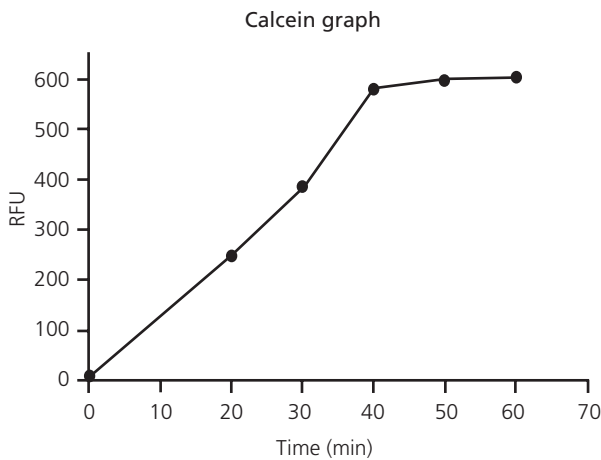


Fig. 1.- Calcein graph. After administration calcein enters the cell, and fluorescence will only be obtained in viable cells. The curve becomes stable at 40 minutes, so measurement data for calcein cellular intake were obtained from this time on. RFU: Relative fluorescence units. *Curva de calceína. Tras administrar la calceína, esta penetra en el interior celular, y sólo en aquellas células viables se emitirá fluorescencia. Se observa cómo dicha curva entra en meseta a los 40 minutos, por lo que la lectura de su actividad se ha realizado a partir de este tiempo. UFR: unidades de fluorescencia relativa.*

rescence is reached after 40 minutes of incubation, so these were the conditions maintained for further studies.

Cell viability after 48 hours of treatment with AS-ODN

In figure 2 we show viability after 48 hours of treatment with AS-ODN depending on concentration:

1. 20 µM concentration treatment:
 - AS-KRAS decreases cell viability by 41%.
 - Telp5 decreases cell viability by 26%.
 - Combined AS-KRAS and Telp5 decrease cell viability by 99.6%.
 - ISIS decreases cell viability by 14.52%.
2. 10 µM concentration treatment:
 - AS-KRAS decreases cell viability by 32.3%
 - Telp5 decreases cell viability by 0.6%.
 - Combined AS-KRAS and Telp5 decrease cell viability by 34.65%.
 - ISIS decreases cell viability by 6%.
3. 5 µM concentration treatment:
 - AS-KRAS decreases cell viability by 32%.
 - Telp5 decreases cell viability by 0%.
 - Combined AS-KRAS and Telp5 decrease cell viability by 30%.
 - ISIS decreases cell viability by 8%.
4. 1 µM concentration did not affect cell viability.

The effect of AS-ODN treatment at peak concentration for 48 hours is shown in figure 3.

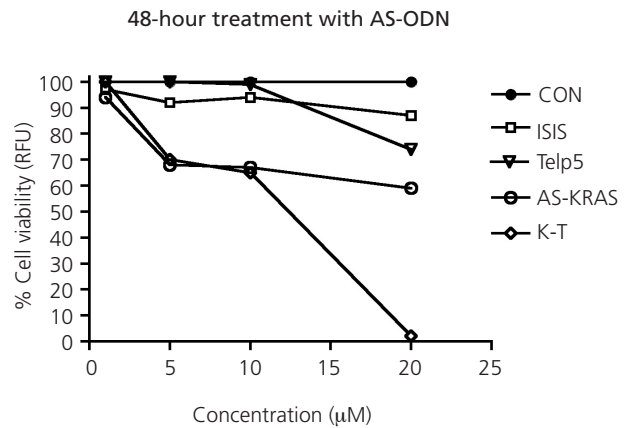


Fig. 2.- Effects of 48-hour treatment with AS-ODNs on cell viability. Viability is expressed as relative fluorescence units (RFU). The combination of anti-k-ras and antitelomerase at 20 µM concentrations produced a fluorescence emission lower than 1% (p = 0.0001). CON = control group, cells without treatment; Telp5 = antitelomerase; AS-KRAS = anti-K-ras; K-T = combination of anti-K-ras and antitelomerase; ISIS = anti-k-ras Isis 6957.

Efectos de la aplicación durante 48 horas de ODN-AS sobre la viabilidad celular en función de las concentraciones empleadas. La viabilidad se expresa en unidades de fluorescencia relativa (UFR). La combinación de tratamientos anti K-ras y antitelomerasa en concentraciones 20 µM, se asocia a una emisión de fluorescencia inferior al 1% (p = 0.0001). CON = control, sin tratamiento; Telp5 = anti-telomerasa; AS-KRAS = anti K-ras y K-T = tratamiento combinado (anti K-ras y antitelomerasa); ISIS= anti k-ras Isis 6957.

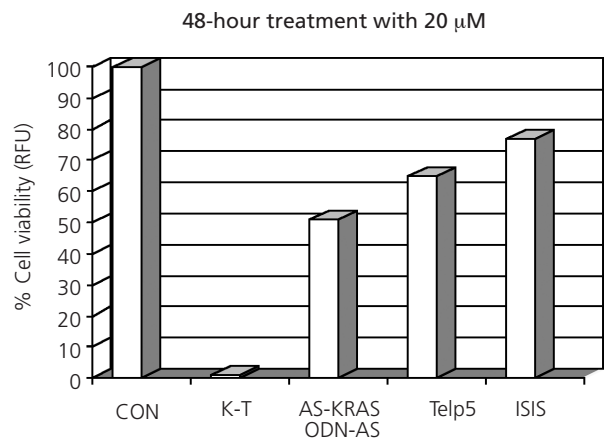


Fig. 3.- 48-hour treatment with the highest concentration of AS-ODN. Cells without treatment (control group; CON) show 100% viability. Combining anti-k-ras and antitelomerase, ODN viability is decreased to < 1%.

Efecto de los ODN-AS con la máxima concentración aplicada durante 48 horas. Las células sin tratamiento o grupo control (CON), presentan 100% de viabilidad. Con el tratamiento combinado anti K-ras y antitelomerasa, la viabilidad celular se reduce a menos de 1%.

Cell viability after 72 hours of treatment with AS-ODN

In figure 4 we show how cell viability decreases after 72 hours of treatment with AS-ODN:

1. *10 µM concentration treatment:*

—AS-KRAS decreases cell viability by 56%.

—Telp5 decreases cell viability by 4%.

—Combined AS-KRAS and Telp5 decrease cell viability by 99.8%.

—ISIS decreases cell viability by 28.5%.

2. *5 µM concentration treatment:*

—AS-KRAS decreases cell viability by 55.57%.

—Telp5 decreases cell viability by 54.68%.

—Combined AS-KRAS and Telp5 decrease cell viability by 63.07%.

—ISIS did not affect cell viability.

3. *1 µM concentrations did not affect cell viability.*

Combined treatment with AS-KRAS and Telp5 caused a 100% mortality of tumoral cells.

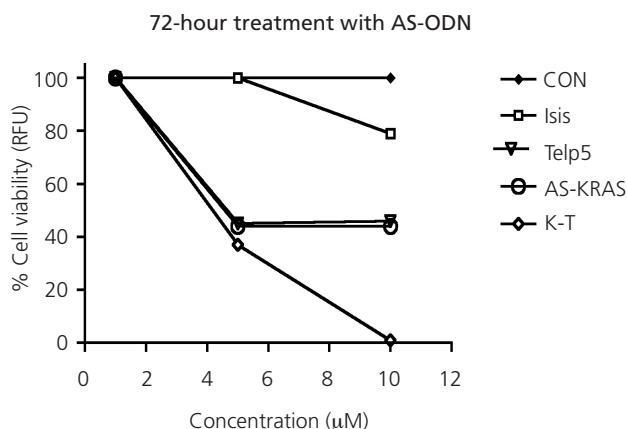


Fig. 4.- Effects of 72-hour treatment with AS-ODNs on cell viability. Viability is expressed as relative fluorescence units (RFU). The combination of anti-k-ras and antitelomerase at 10 µM concentrations produces a fluorescence emission lower than 1% ($p < 0.0001$). The 1 µM dose had no statistical significance ($p < 0.05$) for any applied treatment. CON = control group; cells without treatment; Telp5 = antitelomerase; AS-KRAS = anti-K-ras; K-T = tratamiento combinado (anti K-ras y antitelomerasa); ISIS = anti k-ras Isis 6957.

Efectos de la aplicación durante 72 horas de ODN-AS sobre la viabilidad celular en función de las concentraciones empleadas. La viabilidad se expresa en unidades de fluorescencia relativa (UFR). La combinación de tratamientos anti K-ras y antitelomerasa en concentraciones 10 µM, se asocia a una emisión de fluorescencia inferior al 1% ($p < 0,0001$). La dosis 1 µM no fue estadísticamente significativa ($p < 0,05$) para ningún tratamiento aplicado. CON = control, sin tratamiento; Telp5 = antitelomerasa; AS-KRAS = anti K-ras y K-T = tratamiento combinado (anti K-ras y antitelomerasa); ISIS = anti k-ras Isis 6957.

DISCUSSION

In our study we tested the effect on cell viability of antisense therapy with oligodeoxiribonucleotides blocking

the k-ras oncogene and telomerase gene in a human colorectal cancer line (SW480). Our results show that independent treatment with each AS-ODN can exert partially inhibiting effects (approximately 50%) on cell proliferation. Nevertheless, a combination of anti-k-ras and antitelomerase has a remarkable synergy that leads to a complete loss of tumoral cell viability ($> 99.5\%$).

In vitro cytotoxicity tests are of great importance in the development of new biological agents with cytotoxic activity, especially if those procedures are of high sensitivity and avoid the use of conventional nuclide (usually Cr^{51}) protocols. In our study we used a cytotoxicity test based on microplate cytofluorometry. It is based on fluorochrome calcein with chemically concealed fluorescence. After active cellular metabolization fluorescence is recovered, and this metabolization can only be done by viable cells.

Cytotoxicity studies with fluorometry show that all AS-ODNs tested achieved significant reductions in cell viability. Nevertheless, relevant differences in efficacy showed up. Of the two AS-ODNs tested against the k-ras oncogene, ISIS (designed to link mRNA's 5' terminal end of k-ras) showed a 30% inhibiting effect on cell viability. In equal conditions, AS-KRAS (designed to bind exon 1 of k-ras oncogene) had a higher inhibiting activity (55%). In both cases the maximum effect seems to have been reached, because after 72 hours of treatment period with 5 µM ODN concentrations viability does not decrease with higher concentrations. If we compare our results with those from other groups, a 20% viability reduction has been described by blocking k-ras with a cationic lipid leaded ODN after a 48-hour treatment period (17). Guan Chen's team achieved 70% of cell proliferation inhibition after 72 hours of treatment with anti k-ras ODN (14). In this same study ISIS 6957 was tested, and it only yielded 26% of inhibition in cellular growth with 400 nM after 72 hours of treatment. In their experiment they used a human lung fibroblast model and bladder cancer cells, so results are only partially comparable to ours, as we used another cell type for the tests.

Cell viability results with our antitelomerase ODN were similar in power and efficacy to those obtained with our anti-k-ras ODNs. Both 5 and 10 µM concentrations produced decreased viability in similar rates, which suggests that these doses are most specifically effective for Telp5.

Other studies (18-21) proved the possibility of blocking the telomerase gene with a phosphorotioate ODN. Herbert (22) demonstrated a 50% viability inhibition with 1 nanomolar lipid-vehiculized treatment, and also a viability reduction with non-lipid-vehiculized micromolar concentrations. In Herbert's experiment their antitelomerase ODN also binds the telomerase hTR subunit, but results are not comparable to ours for cultures were of breast cancer epithelial cells. Elayadi et al. (23) described telomerase gene blockade with a phosphorotioate ODN in a Jurkat T lymphocyte (leukemia) cell line. After 36 hours of treatment with 0.25 µM cellular

activity decreased in 27%. A 2 μ M dose decreased viability by 65%. ODNs were lipid-vehiculized with lipoplectamine. Recently, Wong et al. (24) published their results using ODNs that link the telomerase hTR subunit in CRC, and they obtained varied results on viability reduction depending on the type of cell line tested (12-37%).

Our results in antisense gene therapy point out that AS-ODNs designed by our group are some of the most powerful ever described. Moreover, AS-KRAS acts earlier in time than Telp5. An explanation for this could be the different metabolic pathways followed by each AS-ODN. Nevertheless, what is of great relevance is the fact that our combined anti-k-ras and antitelomerase treatment has a synergic effect that leads to an inhibition of cell viability reaching > 99.5%. This opens an attractive alternative pathway in antisense therapy, specially considering that those results may be improved using transfection vectors. This, together with the improvement of cell transfection with AS-ODN (with a subsequent reduction in therapeutical dose) suggests the ability to simultaneously introduce combinations of AS-ODNs in cancer cells. Moreover, our results have been expressed as cell viability reduction rates. This is a good approximation to explain antisense therapy effects, but it would be desirable to quantify telomerase and k-ras expression, as well as treatment-derived proteins in further studies.

In summary, gene therapy with antisense oligonucleotides has been shown to be effective in our experience, especially the combination of anti k-ras and antitelomerase treatment, both implicated in CRC cell viability. *In vivo* applicability, usefulness and secondary effects need to be further established. Further studies will be needed to evaluate the *in vivo* efficacy of these AS-ODNs and their combinations using transfection vectors for the improvement of clinical applications.

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