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siRNA is not more Effective than a First Generation Antisense Oligonucleotide when Directed against EGFR in the Treatment of PC-3 Prostate Cancer

PAULUS TSUI[1], MARVIN RUBENSTEIN[1,2] and PATRICK GUINAN[1,3,4]

[1]Division of Cellular Biology, Hektoen Institute for Medical Research;
Departments of [2]Biochemistry and [3]Urology, Rush University Medical Center;
[4]Department of Urology, University of Illinois at Chicago, Chicago, IL, 60612, U.S.A.

Abstract. *siRNA specifically directed against the epidermal growth factor receptor (EGFR) was compared to the previously described and effective MR2 oligo (also specific for EGFR) in a total of six comparative studies utilizing the chemotherapeutic agents Taxol, cisplatin and Cytoxan and the PC-3 prostate tumor line. When Taxol was administered in combination with either MR2 or siRNA, the MR2 was significantly more effective (p=0.000277) against PC-3 cells incubated for 24 h in their presence. In a sequential study in which a 24-h Taxol treatment was followed with either MR2 or siRNA for an additional 24 h, or in the reverse order where MR2 or siRNA was followed by Taxol, no significant differences were found. When cisplatin was similarly administered in combination with either MR2 or the siRNA, no significant differences in inhibition were found. In a subsequent study, in which a 24-h treatment with either MR2 or siRNA was followed by cisplatin for an additional 24 h, again no significant differences were found. Lastly, in a series of sequential administrations including Cytoxan the following was found. PC-3 cells treated for 24 h with Cytoxan followed by either MR2 or siRNA produced similar inhibition. When the cycle was reversed, with MR2 or siRNA treatment followed by Cytoxan, both treatments were again inhibitory, however the initial treatment with MR2 was significantly more effective (p=0.026095). We conclude that, although siRNA against EGFR has efficacy against the PC-3 line when administered either alone vs untreated controls, with Taxol vs Taxol alone, or with cisplatin vs cisplatin alone, it is not more effective than the better characterized MR2 oligo.*

Correspondence to: Dr. Marvin Rubenstein, Chairman-Division of Cellular Biology, Hektoen Institute for Medical Research, 2100 W. Harrison Street, Chicago, IL, 60612, U.S.A. Tel: 312-864-4621, Fax: 312-948-2509, e-mail: DrMarv@Prodigy.net

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Antisense oligonucleotides (oligos), directed against a variety of specific proteins, are well described as agents useful for treating experimental prostate tumor models. In efforts to enhance their efficacy, we have evaluated combinations of traditional chemotherapeutics with specific oligos, which have previously demonstrated significant improvements in inhibiting prostate tumor cell growth when administered in proper sequence.

In an initial study, we concluded that pretreatment with an antisense oligo (an oligodeoxynucleotide [ODN]) directed against transforming growth factor-alpha (TGF-α) primes PC-3 prostate cancer cells for significantly enhanced treatment with Taxol (1). In subsequent studies, we found that combination therapy which included Cytoxan, mitoxantrone, and the platinates carboplatin, cisplatin and oxaliplatin, also was efficacious (2).

However, many investigators now claim that oligo-like derivatives, such as siRNA (3), might have even greater effectiveness (4, 5). In an effort to compare the effectiveness of siRNA *versus* effective but traditional first generation (phosphorothioated) oligos, we directly compared their effectiveness in combination therapy using the PC-3 *in vitro* cell line.

The MR2 ODN directed against the epidermal growth factor receptor (EGFR) was chosen for comparison with an siRNA developed against the same target protein. MR2 has been extensively evaluated and has demonstrated significant efficacy against LNCaP and PC-3 prostatic tumor models, where it has inhibited both *in vitro* (6) and *in vivo* growth (7), as well as produced hemorrhagic necrosis following intratumoral inoculation (7). When similarly evaluated, the UACC 893 breast tumor line (8) and the T98G glioblastoma (9) behaved similarly. On March 11, 1997, the MR2 ODN was issued US patent #5,610,288 for the treatment of hormone-insensitive tumors.

siRNA specific for the epidermal growth factor receptor (EGFR) was compared to the previously

Table I. *Inhibition of PC-3 Cell growth produced by oligos and chemotherapeutics.*

Control	Taxol	Taxol+MR2	Taxol+siRNA	Taxol/MR2	Taxol/siRNA	MR2/Taxol	siRNA/Taxol
788.4±75.7	366.9±35.3	275.9±27.1	331.1±23.3	564.1±37.2	609.9±61.8	496.9±37.2	480.4±38.0
		p=0.000277 MR2 < siRNA		p=0.08903 NS		p=0.394733 NS	

Control	Cisplatin	Cisplatin+MR2	Cisplatin+siRNA	MR2/Cisplatin	siRNA/Cisplatin
725.8±74.2	308.3±32.6	269.1±64.8	204.1±79.5	243.8±88.9	256.1±43.8
		p=0.060466 NS		p=0.699196 NS	

Control	Cytoxan	Cytoxan/MR2	Cytoxan/siRNA	MR2/Cytoxan	siRNA/Cytoxan
457.1±87.8	179.1±13.8	200.7±41.5	189.1±19.1	281.3±41.1	331.9±33.6
		p=0.482385 NS		p=0.026095 MR2 < siRNA	

described and effective MR2 oligo in a total of six comparative studies utilizing the chemotherapeutic agents Taxol, cisplatin and Cytoxan.

Materials and Methods

Oligonucleotides. Oligodeoxynucleotide (ODN) MR2, directed against the epidermal growth factor receptor (EGFR), has been described previously (2, 7) and is covered by US Patent #5,610,288. Briefly, it is a first generation 39 mer, terminally phosphorothioated (3 bases at 5' and 3' ends) ODN having sequence complementarity about the AUG initiation codon. It was obtained from Qiagen (Valencia, CA, USA). siRNA, also directed against EGFR, was purchased from SuperArray Bioscience Corporation (Frederick, MD, USA).

Chemotherapeutics. Paclitaxol (Taxol) and cyclophosphamide (Cytoxan) were purchased from LKT Labs (St. Paul, MN, USA). Cisplatin was obtained from a hospital pharmacy.

Solutions. Antisense ODNs were prepared in serum-free OPTI-MEM culture media (Invitrogen Inc., Carlsbad, CA, USA) to a stock concentration of 625 μM. For each well, 1.6 μl of Lipofectin reagent (Life Technologies Inc., Gaithersburg, MD, USA) (1 mg/ml) was diluted in 18.4 μl of OPTI-MEM medium and incubated at room temperature for 30 minutes. Two solutions (ODN and Lipofectin) were then combined in equal volumes (20 μl), mixed and incubated at room temperature for another 15 minutes. The volume was then adjusted to 200 μl with OPTI-MEM for each well and Lipofectin-oligo complexes were added to wells with cells. The final Lipofectin concentration was 8 μg/ml/well.

Cell culture conditions and determination of growth. PC-3 cells were maintained in F-12 media containing 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin in a 5% CO_2 incubator. Four days prior to the addition of ODNs, 1 X 10^4 PC-3 cells were added, in a total 200 μl volume of media, to each depression of a 96-well plate and incubated at 37˚C in a CO_2 incubator. On the day of transfection, the following solutions were prepared:

A) 1 μl of buffer containing either siRNA or a control substance was added to 50 μl of OPTI-MEM and gently mixed. One dilution was made for each well.
B) 1 μl of Lipofectin was diluted in 50 μl of OPTI-MEM and mixed gently for 5 minutes at room temperature.
C) ODN (MR2 or siRNA) dilutions were mixed with 50 μl of Lipofectin and gently mixed for 20 minutes at room temperature.
D) 100 μl of the Lipofectin and ODN mixture was added to 100 μl of F-12 medium and mixed.

Chemotherapeutics were then added to the following concentrations: Taxol, 5 nm; cisplatin, 20 nm; Cytoxan 40 nm. The cells were then incubated for 24-48 h before solutions were aspirated and re-incubated for an additional 48 h in 200 μl of F-12. Cell counts were determined following the addition of WST-1 reagent to each well and, after 2 h, the color intensity was measured by a microplate reader at a wavelength of 450 nm, using a reference wavelength of 650 nm. The values obtained were determined after the subtraction of paired blank samples from the experimental wells and were multiplied by a constant to give whole integers for analysis.

Statistics. Microsoft Excel software was utilized to calculate means and standard deviations and Students *t*-tests were used to determine significance.

Results

In the first series of experiments (Table I), PC-3 cells were incubated in the presence of Taxol and the two oligo compounds. The cultures were treated either with Taxol alone, Taxol in combination with MR2 or siRNA for 24 h, Taxol for 24 h followed by either the MR2 or the siRNA oligos for a second 24-h period, or the reverse order of 24-h incubations including either the MR2 or siRNA oligos, followed by Taxol. Control values were 788±76, and treatment groups of Taxol alone, Taxol in combination with MR2, Taxol in combination with siRNA, Taxol followed by MR2, Taxol followed by siRNA, MR2 followed by Taxol and siRNA followed by Taxol had respective values of 367±35, 276±27, 331±23, 564±37, 609±62, 497±37 and 480±38. While each treatment inhibited growth of PC-3 cells relative to the control, there was no significant difference between MR2 and siRNA treatments followed by Taxol. However, the combination treatment of Taxol with either MR2 or siRNA showed a significant advantage using MR2 ($p=0.000277$).

In the second series of experiments (Table I), PC-3 cells were incubated in the presence of cisplatin and the two oligo compounds. The cultures were treated either with cisplatin alone, cisplatin in combination with MR2 or siRNA for 24 h or sequentially with MR2 or siRNA followed by cisplatin for a second 24-h period. Control values were 726±74, and treatment groups of cisplatin alone, cisplatin and MR2, cisplatin and siRNA, MR2 followed by cisplatin and siRNA followed by cisplatin had respective values of 308±33, 269±65, 204±80, 244±89 and 256±44. While each treatment inhibited growth of PC-3 cells relative to the control, there was no significant difference between combinations of cisplatin with either MR2 or siRNA or sequential treatments with MR2 or siRNA followed by cisplatin.

In the final series of experiments (Table I), PC-3 cells were incubated sequentially in the presence of Cytoxan and the two oligo compounds. Cultures were treated either with Cytoxan alone, with Cytoxan for 24 h followed by either the MR2 or the siRNA oligos for a second 24-h period, or initially with a 24-h incubation including either the MR2 or siRNA oligos, followed by a final 24 h with Cytoxan. Control values were 457±88, and treatment groups of Cytoxan alone, Cytoxan followed by MR2, Cytoxan followed by siRNA, MR2 followed by Cytoxan and siRNA followed by Cytoxan had respective values of 179±14, 201±42, 189±19, 281±41 and 332±34. While each treatment inhibited the growth of PC-3 cells relative to the control, there was no significant difference between Cytoxan followed by MR2 or siRNA treatments. When the cycle was reversed with MR2 or siRNA treatment followed by Cytoxan, both treatments were again similarly inhibitory, however the initial treatment with MR2 was significantly more effective ($p=0.026095$).

We conclude that, although siRNA against EGFR has efficacy against the PC-3 line when administered either alone *vs* untreated controls, with Taxol *vs* Taxol alone, or with cisplatin *vs* cisplatin alone, it is not more effective than the better characterized MR2 oligo.

Discussion

Antisense oligos consist of synthetic single strands of nucleic acids which are complementary in base sequence to mRNA. Their biological activity is based primarily on their hybridization to mRNA and the failure of that mRNA to be translated through a variety of mechanisms. These include destruction of the duplex by RNAse H, triplex formation, protein binding interactions with growth factors which remove the oligo from action (10), or other non-specific mechanisms which may not be dependent upon mRNA expression (11). The original first generation oligos were limited by half-life, inability to cross the blood brain barrier, poor solubility and some toxicity. To extend the half-life and inhibit nuclease activity, phosphorothioation was employed. Other limitations have been overcome by creating second and third generation antisense oligos with modified backbone, sugars or morpholino formulations (review in 5). Antisense technology has also produced a variety of additional derivative forms, including ribozymes and triplexes (5).

A perceived revolution in the antisense field began several years ago with the identification and description of RNA interference (RNAi) by Mello and Fire (3). This work was derived from previous reports of transgene splicing in plants and also of dsRNA inhibiting gene expression in the worm *C. elegans.* Small interfering RNAs (siRNAs) are 21-26 mer in length. They form an RNAi-induced silencing complex with a protein, which then aids in the unwinding of dsRNA, permitting antisense strand binding with its mRNA target. It is widely thought that siRNAs are powerful alternatives to antisense oligos, although they are also subject to many of the same delivery, specificity and cost problems (5).

We conclude that, although siRNA against EGFR has efficacy against the PC-3 line, it is not more effective than the better characterized MR2 oligo. Furthermore, we suspect that siRNA would provide no advantage in its ability to be specifically delivered, and recent studies show that it is rapidly degraded in the blood and tissues if not chemically modified (12).

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