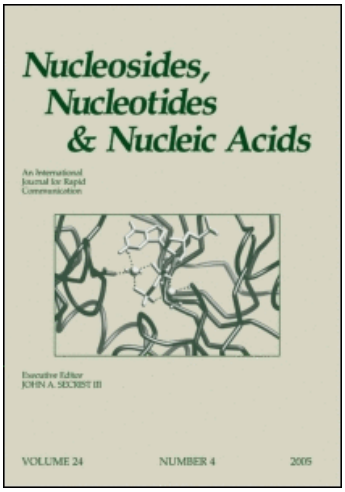

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ANTISENSE OLIGONUCLEOTIDE CONJUGATES WITH PHOTOSENSITIZERS - AN UPDATE

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INTRODUCTION:

The fascinating aspect of using oligonucleotide conjugates for therapeutic purposes is to combine the activity of known drugs with the addressing effect of the oligonucleotide and its potential of inhibiting protein synthesis. In this sense, some interest has been given to oligonucleotide conjugates with photosensitizers[1], in particular with porphyrins.

Experiments with oligonucleotide porphyrin conjugates discribed in the literature[2] have their focus on the molecular aspect, demonstrating the potential of site-specific DNA damage. Porphine derivatives are also valuable tools for cell biology, since they are able to produce a phototoxic effect in cell systems, which can be triggered by irradiation. This phototoxic effect has been the basis for photodynamic therapies.

Some years ago Rück et al.[3] have described the advantages of using hydrophilic porphyrins to achieve such a phototoxic effect in cell cultures on irradiation with laser light. On the basis of these experiments we previously prepared a conjugate of meso-(tetra-4-carboxyphenyl-) porphine ($TPPC_4$) to an oligonucleotide antisense to actin mRNA[4]. With this compund we observed a significant decrease of the concentration necessary for producing phototoxicity on incubation in RR1022 rat epithelial cell

cultures, which suggests a synergistic action of the porphyrin and oligonucleotide moieties.

In recent studies we have addressed some questions related to the reasons of this „synergistic effect": Would the uptake of the porphyrin be enhanced, i.e. the intracellular concentration be (locally) increased through the affinity of the oligonucleotide part to mRNA? If so, it would have to be proven that enhanced uptake and phototoxicity would be correlated with the length and sequence of the oligonucleotide substituent.

MATERIALS AND METHODS

Chemicals and Materials: Reagents, solvents and supports for the chemical synthesis of the oligonucleotide porphyrin and fluorescein conjugates were obtained from Glen Research (Stirling, USA), Fluka (Buchs, Switzerland), Carl Roth GmbH (Karlsruhe) and E. Merck (Darmstadt, Germany). Amino modifier phosphoramidites, were purchased from Glen Research. $TPPC_4$ was a kindly provided by W. S. L. Strauss, Institut for Laser Applications in Medicine, Ulm.

High performance liquid chromatography was done on a Waters Associates 6000A HPLC using a Waters 440 absorbance detector and Value Chrom TM chromatography software. Columns LichroCART 125-4 from E. Merck were filled with LiChrospher 100 PR-18 (5μm).

Solid phase oligonucleotide synthesis and attachment of photosensitizers were done in a Pharmacia Gene Assembler 4 Primers, according to the procedure published previously[4]. Attachment of fluorescein was done on the polymer support by reacting the 5'- end of the oligonucleotide chains with fluorescein phosphoramidite (Fluor Prime™, Pharmacia). After completion the conjugates were deprotected and cleaved from the support by ammonia treatment (55°, overnight), then purified by HPLC using a linear gradient of 0.01M ammonium acetate (pH7) + 0 - 50% acetonitrile.

Cell cultures: The virus transformed rat epithelial cell line RR 1022 (ATCC-no. CCL 47) was obtained from Flow Laboratories (Meckenheim, Germany). The cells were cultured in Dulbecco's modified Eagle medium (Serva, Heidelberg) buffered with sodium hydrogen carbonate, to which were added 100 U/l penicillin, 100 ug/l streptomycin and 600ug/l L-glutamine. 10% fetal calf serum were supplemented during cultivation (ca.

72h, 37°). The cells were trypsinized, harvested by centrifugation, the pellet resuspended in the medium and used as a stock suspension for slide cultures.

Fluorescence microscopy: The subcellular localization of the oligonucleotide conjugates was evaluated using a laser scanning microscope (LSM 410 invert, Zeiss, Germany). Confocal fluorescence images were obtained by exciting the cells with an internal HeNe laser (633 nm), with detection of the fluoresence above 665 nm with a photomultiplier (beam splitter FT 655 nm, long-pass filter RG 665 nm). The fluorescence distribution was recorded with the red channel of the microscope. Simultaneously, the phase contrast images were detected with the green channel. A 40 x magnification phase contrast objective lens (aperture, 0.75 mm) was used together with a zoom factor of two.

Induction of phototoxic effect: The cell cultures were grown at 37°C on microscopic slides, incubated with 10 μmol of each of the oligonucleotide conjugates, alternatively with 10 μmol $TPPC_4$ as a control, at 37°C for 24 h and then washed with PBS. After washing, individual cells of each slide were irradiated for 2 min with laser light of wavelenght 633 nm and than screened for morphological changes. One second of illumination corresponded to an irradiation of 1.7 J/cm^2.

RESULTS

Preparation of porphyrin and fluorescein substituted oligonucleotides

Using essentially the same system as before[4], we synthesized a 27 base oligonucleotide specific to rat epithelial α-actin mRNA and three nested sequences of length 20, 17 and 11 bases (Table 1). The structures of the conjugates are represented in Fig. 1. Additionally, a 20 base non-related sequence was prepared as a control. All sequences contained a 3'-end sense inversion for protection against intracellular exonucleolytic degradation[5]. The conjugation to $TPPC_4$ was done by the solid-phase procedure described before[4].

The support-bound oligonucleotide was 5'-terminated with a C_{12} amino linker by reaction with β-cyanoethoxy-[(N-monomethoxytrityl-)ω-aminododecyloxy]-(N,N-diisopropyl-) phosphoramidite. Subsequently, $TPPC_4$ was attached by coupling with 1-

TABLE 1: Synthetic oligonucleotide conjugates and corresponding target

Oligonucleotide (5'-3')	Sequence
F1	$\mathbf{R}^1$-AGCCTCGTCGTACTCCTGCTTGGTGAT$_{inv.}$
P1	$\mathbf{R}^2$-AGCCTCGTCGTACTCCTGCTTGGTGAT$_{inv}$
F2	$\mathbf{R}^1$-AGCCTCGTCGTACTCCTGCT$_{inv}$
P2	$\mathbf{R}^2$-AGCCTCGTCGTACTCCTGCT$_{inv}$
F3	$\mathbf{R}^1$-AGCCTCGTCGTACTCCT$_{inv}$
P3	$\mathbf{R}^2$-AGCCTCGTCGTACTCCT$_{inv}$
F4	$\mathbf{R}^1$-AGCCTCGTCGT$_{inv}$
P4	$\mathbf{R}^2$-AGCCTCGTCGT$_{inv}$
F5	$\mathbf{R}^1$-CTAAGATGATGCAGAAGTAT$_{inv}$
P5	$\mathbf{R}^2$-CTAAGATGATGCAGAAGTAT$_{inv}$
Target	
rat epithelial α-actin mRNA pos. 952-978	AUCACCAAGCAGGAGUACGACGAGGCU

$\mathbf{R}^1$= Fluorescein-linker (see fig. 1)
$\mathbf{R}^2$= Porphyrin-linker (see fig. 1)
$T_{inv.}$ = -3'-3'-T

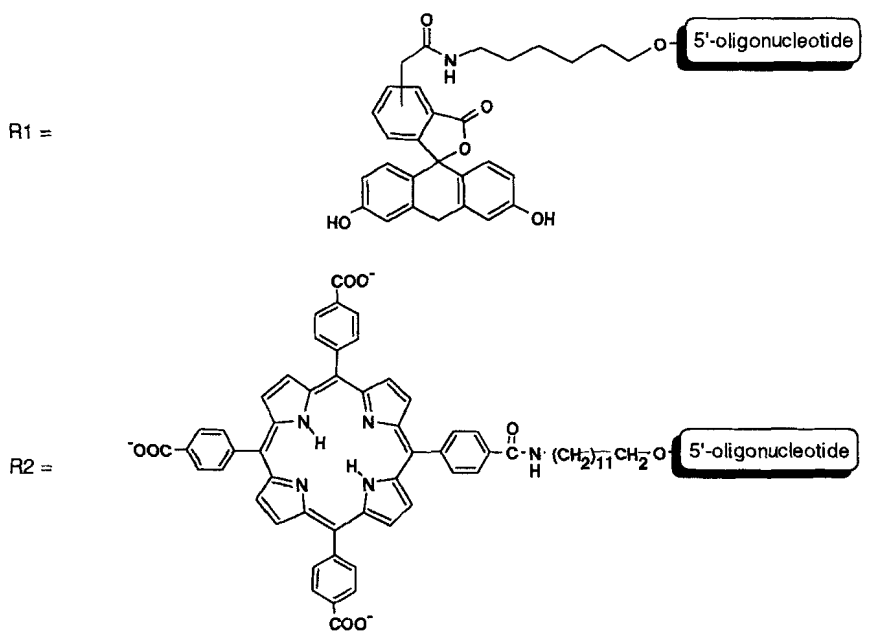

FIG. 1: Structures of oligonucleotide conjugates.

ethyl-3-(3-dimethylaminopropyl-) carbodiimide. The best yields of conjugation (ca. 50%) were obtained using the longer linker.

Structural proof for conjugation to the porphyrin moiety was obtained by UV/vis spectroscopy through the appearance of an additional Soret band at 427 nm.

Similarly, fluorescein was attached to the oligonucleotides shown in Table 1 via a C_6-amino linker. Gel electrophoresis showed essentially quantitative conversion with formation of a single spot corresponding to a fluorescent and UV-absorbing product.

Celluar uptake and intracellular localization of oligonucleotide conjugates

RR 1022 rat epithelial cells were incubated with 10 μmol of each of the oligonucleotide conjugates, alternatively with 10 μmol $TPPC_4$ as a control, at 37° for 24 h. In order to avoid undesired photoreactions, the cultures were kept in the dark.

Cellular uptake was visualized by confocal fluorescence microscopy (Fig. 2). A series of experiments done with fluorescein oligonucleotides showed high uptake for the 20 base (Fig 2B), internalization in lesser amount also for the 27 base oligonucleotide conjugate (Fig. 2A). Oligomers F3 (Fig. 2C) and F4 (17 and 11 bases) were poorly internalized, and no intracellular fluorescence was found for the conjugate prepared with a 20base sequence not related to actin mRNA (Fig.2D). Cells, that had lost their viability, would show fluorescence uptake regardless of length and sequence of the oligonucleotide.

Similar internalization results were obtained with porphyrin oligonucleotide conjugates, although intracellular fluorescence was not so clear to see, due to lower intensity.

In line with our previous observations[4], intracellular fluorescence, if present in viable cells, was seen to accumulate in the cytoplasm.

Phototoxic effect of oligonucleotide porphyrin conjugates

After incubation with oligonucleotide porphyrin conjugates and removal of extracellular fluorescence the phototoxic effect on the cell cultures was tested on irradiation with laser light of $\lambda = 633$ nm. Using the 20 base oligonucleotide conjugate P2, the loss of viability of the cells was clearly demonstrated by a change of appearance and contours (Fig. 3A,B). The 27 base compound P1 showed no cytotoxicity under the experimental conditions chosen here (Fig.4A,B). Phototoxicity was not observed - and

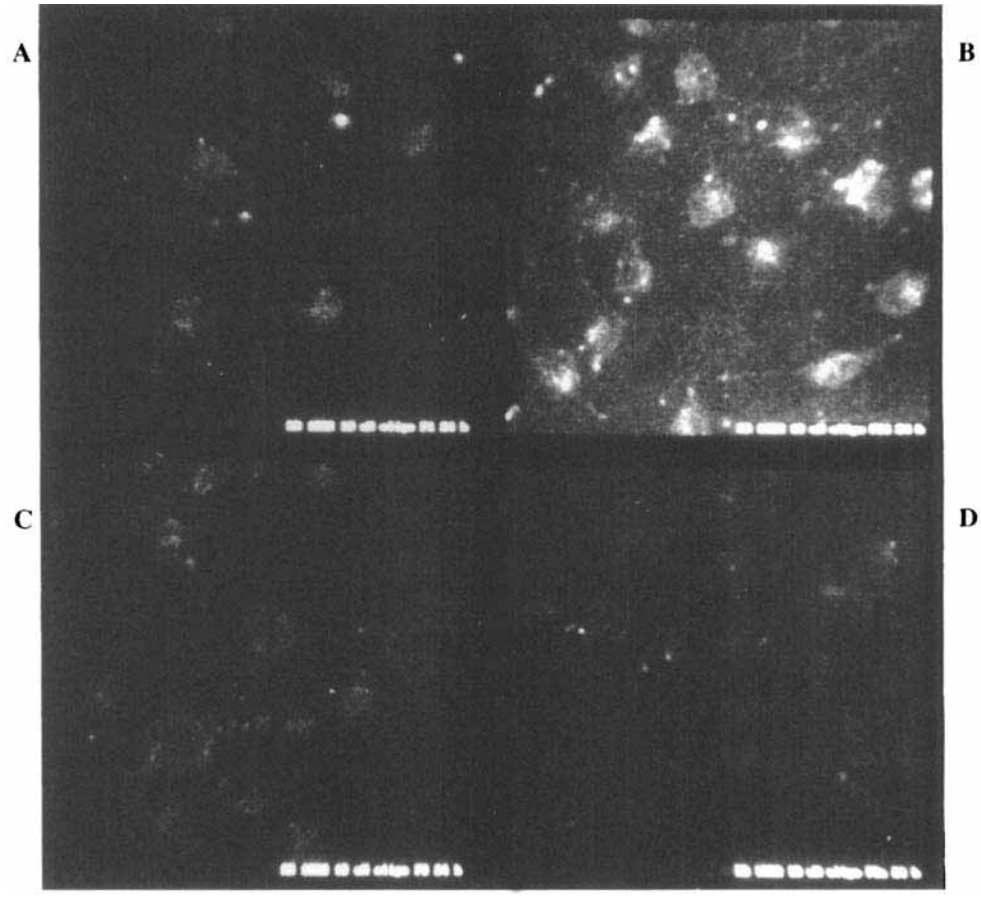

FIG. 2: Distribution of fluorescence localization in RR 1022 ephitelial cells incubated for 24 h with 10 μM fluorescein oligonucleotides of length (**A**) 27 Nt, (**B**) 20 Nt, (**C**) 17 NT, (**D**) actin mRNA non-related sequence.

also not expected - for all other porphyrin conjugates, as well as for the controls, since we had previously not seen significant uptake of these compounds (Fig. 5A,B and 6A,B). Change of morphology due to a phototoxic effect was also not observed in experiments using $TPPC_4$ alone under the same conditions.

DISCUSSION

Novel conjugates of fluorescein and $TPPC_4$ to oligonucleotides of chain length 27, 20, 17 and 11 bases were prepared by the solid phase method described earlier[1]. The

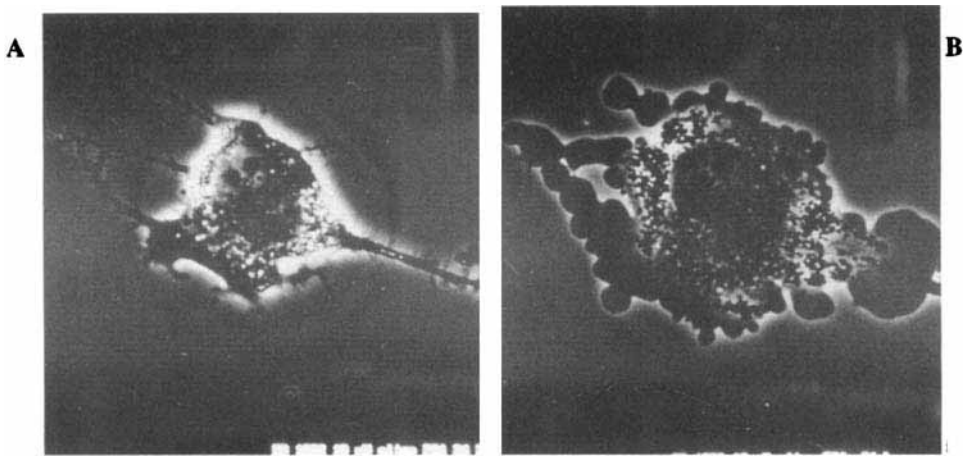

FIG. 3: Phase contrast microscopy of RR 1022 ephitelial cells incubated for 24 h with 10 μM $TPPC_4$-oligonucleotide P2 before (**A**) and 2 min after (**B**) light exposure.

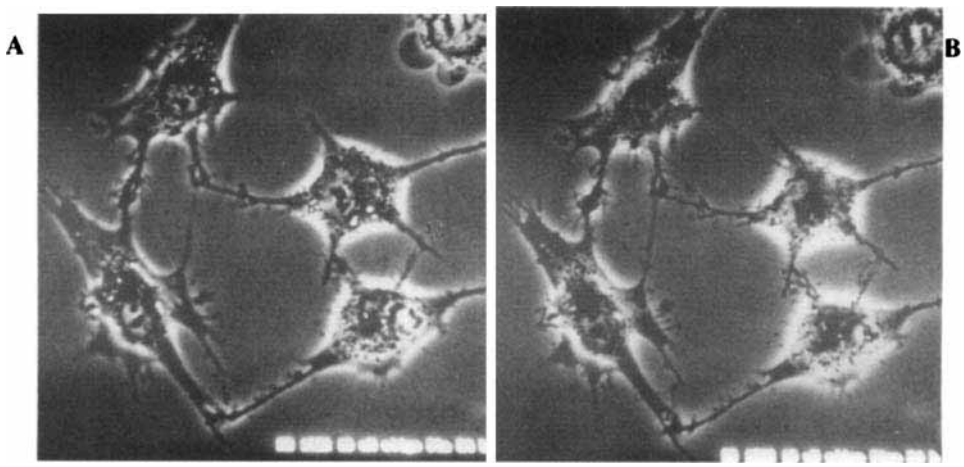

FIG. 4: Phase contrast microscopy of RR 1022 ephitelial cells incubated for 24 h with 10 μM $TPPC_4$-oligonucleotide P1 before (**A**) and 2 min after (**B**) light exposure.

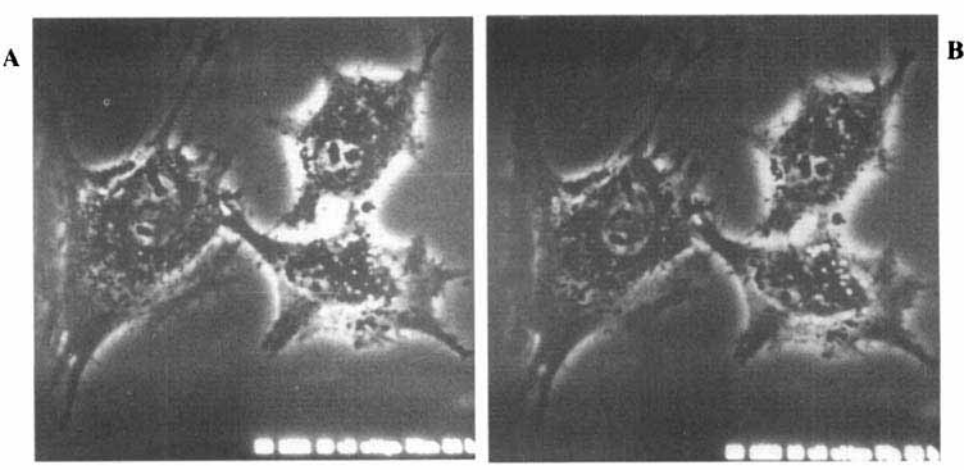

FIG. 5: Phase contrast microscopy of RR 1022 ephitelial cells incubated for 24 h with 10 μM $TPPC_4$-oligonucleotide P3 before (**A**) and 2 min after (**B**) light exposure.

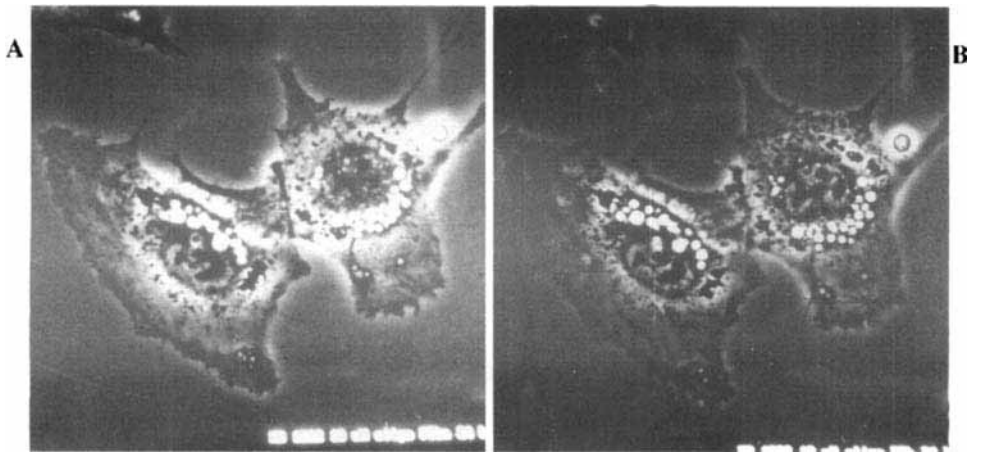

FIG. 6: Phase contrast microscopy of RR 1022 ephitelial cells incubated for 24 h with 10 μM $TPPC_4$-oligonucleotide P5 before (**A**) and 2 min after (**B**) light exposure.

oligonucleotide sequences were antisense to segments of rat actin mRNA. Internalization and phototoxic effect of these conjugates were investigated in cultures of RR1022 cells, which may be assumed to show high-level expression of actin. The results of our studies corroborate our ealier finding, that the conjugate accumulates in the cytoplasm[4] and clearly show a dependence of cellular uptake on length and sequence of the oligonucleotide moiety.

Under comparable conditions the fluorescence detected within the cells decreased significantly, when the oligonucleotide length was less than 20 bases. Intracellular fluorescence was also not detected, when the oligonucleotide sequence was unrelated to the target mRNA. This strongly suggests, that the internalization of the fluorescein or porphyrin is enhanced by the addressing property of the oligonucleotide substituent. The phototoxic effect found on irradiation with laser light of 633 nm was found in cultures incubated with 20b oligonucleotide $TPPC_4$ conjugate, but not with conjugates with 17 and 11b. The fact that the 27 base oligonucleotide porphyrin conjugate showed less uptake and did not exhibit phototoxicity under our experimental conditions, has to be further investigated.

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