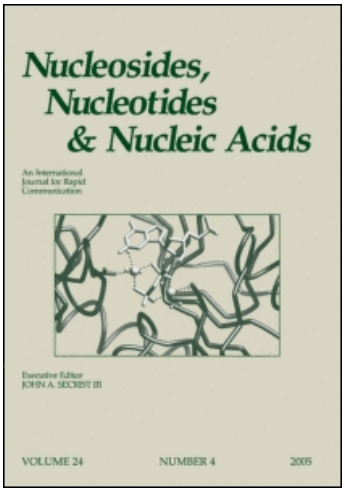

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CELLULAR UPTAKE AND INTRACELLULAR QUANTIFICATION OF FLUORESCENT LABELED T_{20} ME-SATE PROOLIGONUCLEOTIDES

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ABSTRACT

Fluorescein labeled Me-SATE T_{20} models were synthesized. The high uptake of these prooligos in HeLa cells was confirmed by fluorescence microscopy, flow cytometry and by spectrofluorometry.

The antisense oligonucleotides are representing a new class of substances used for several therapeutic targets including viral infections and cancer (1). Unfortunately oligonucleotides are rapidly degraded by nucleases and they exhibit only a weak permeation through biological cell membranes resulting in poor bioavailability. Therefore, a strong need exists for delivery systems, which protect the antisense drugs from enzymatic digestion and provide an enhanced transfection to the cytoplasm of the target cells. Along this line we have been developing the prooligonucleotide approach (2) which consists to transitory mask a part of the negative charge of the phosphodiester linkages to gain prooligos exhibiting higher lipophilicity that yield to an increase of cellular uptake and a better nuclease resistant. Our strategy involves an intracellular carboxyesterase activity to recover the oligonucleotide inside the cell able to interfere with its target in cytoplasm

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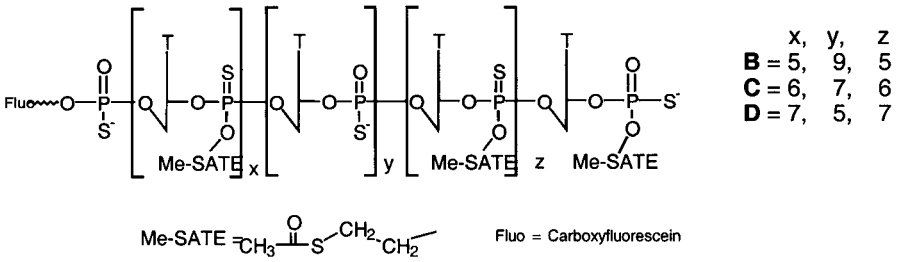

Figure 1. Schematic structure of the Me-SATE T_{20} prooligo models.

or in nucleus. We have shown that prooligos bearing tert-butyl S-acyl-ThioEthyl (tBu-SATE) enzymolabile protection groups are much more taken up by cells than phosphorothioate oligos provided that their displayed enough lipophilicity (3).

Here we present the uptake of different Me-SATE prooligo models (Fig. 1) in HeLa cells by fluorescence microscopy and flow cytometry (FACS), and their intracellular quantification by spectrofluorometry.

Three fluorescein labeled Me-SATE T_{20} prooligo models (**B**, **C** and **D** Fig. 1) bearing 11 to 15 Me-SATE group as well as a control oligo (**A**: Fluo-T_{20} phosphorothioate) were synthesized according to the procedure already published (4). These oligos exhibit an increasing lipophilicity according to the number of SATE groups reflects by their retention time on HPLC, C_{18} reverse phase column (**A**, **B**, **C** and **D**, RT 38, 59, 60 and 65 min respectively).

These oligos were incubated with HeLa cells for 2 h at 37°C in OptiMEM free serum. After cells fixation, fluorescence microscopy showed a rapid and high uptake for the three prooligos while the oligo control **A** was, as expected, poorly taken up (Fig. 2). The uptake efficiency parallels the lipophilicity. The fluorescence was found homogeneously in the cytoplasm and the nucleus without any punctuation. Incubation a 4°C led to only a slight decrease of uptake that indicates uptake proceeds through a passive mechanism.

To gain a relative quantification of the oligo uptake, the prooligo **C** and the control oligo **A** were incubated with HeLa cells at three different concentrations

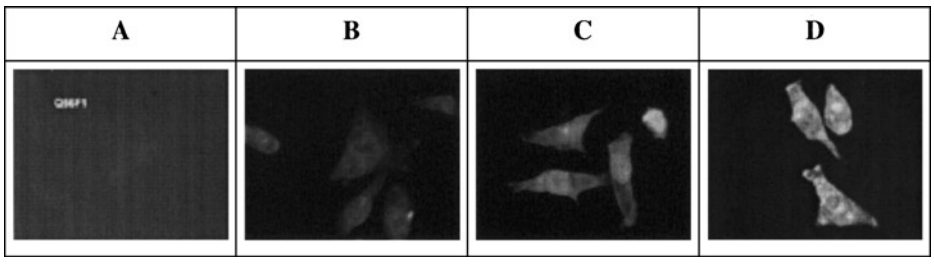

Figure 2. Fluorescence microscopy of oligo **A** and the prooligo **B**, **C** and **D** after incubation with HeLa cells at a 10 μM for 2 h at 37°C in OptiMEM.

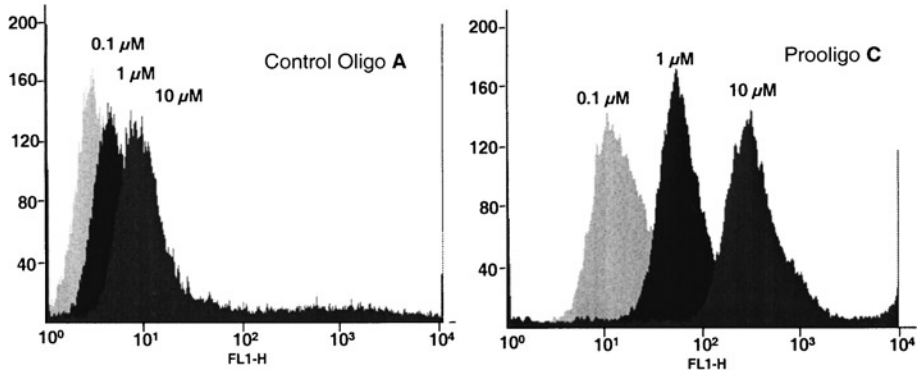

Figure 3. Flow cytometry of oligo A and prooligo C incubated for 30 min with HeLa cells in OptiMEM serum free at 0.1, 1 and 10 μM.

(0.1, 1 and 10 μM). The cells were washed to remove extracellular oligo and the relative fluorescence of the cells was determined by flow cytometry. While the uptake of control oligo **A** was poorly increased when the concentration increases (4-fold between 0.1 to 10 μM) that of prooligo **C** was highly increased (30-fold). Furthermore, the prooligo **C** exhibited a much better uptake at all concentration than **A** (Fig. 3). Indeed uptake of **C** incubated at 0.1 μM was similar to that of **A** incubated at 10 μM.

However we have shown that fluorescein conjugated to a prooligo displays less fluorescence than when it is conjugated to a phosphorothioate oligo (5). This phenomenon prompts us to design another fluorescence method where the two oligos to compare exhibit similar fluorescence properties. Furthermore, we were interested to determine the amount of prooligos into the cells. For that purpose the prooligo **C** and the control oligo **A** were incubated with HeLa cells likewise for the flow cytometry study. The cells were counted and lysed by three freezing defreezing cycles, the resulting mixture was treated with concentrated ammonia to remove all SATE groups that leads to the same type of fluorescein labeled oligo than **A**. After evaporation of the ammonia the residues was dissolved into 120 μL and the fluorescence was measured with a spectrofluorometer. From calibration curves the concentration of each oligo was calculated. For the oligo **A** whatever the concentration used for the incubation (0.1, 1 or 10 μM) the intracellular calculated was around 0.1 to 0.2 μM. In contrast the prooligo **C** exhibited an increase of the intracellular concentration (1–2, 4 and 8–10 μM) when the incubation concentration increased (0.1, 1 and 10 μM respectively).

The three techniques presented here corroborate the high uptake of prooligos. Furthermore complementary studies showed that the Me-SATE groups are effectively hydrolyzed by carboxyesterases present in cell extract. The synthesis of prooligos bearing the four nucleobases are in progress and should give us insights on the efficacy of the prooligos to inhibit gene expression.

ACKNOWLEDGMENT

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