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Evaluation of the cellular uptake of hexitol nucleic acids in HeLa cells

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Two major hurdles to the widespread use of synthetic nucleic acids as drugs are the biological stability of the compounds and efficiency of cellular penetration. Recent advances in the chemistry of nucleic acids has given rise to highly stable derivatives with an anhydrohexitol backbone. This report addresses the cellular uptake of these molecules. We show that the uptake of HNA in the absence of a carrier is very low but HNA is efficiently internalized with a range of transfection reagents.

1. Introduction

Since the initial report on the antisense concept in 1978 [1], considerable progress has been made with the recent FDA approval of the first antisense-based product for a treatment for CMV retinitis [2]. This oligomer belongs to the first generation of antisense molecules, namely those with modified internucleotide linkages, the phosphorothioates. The most fundamental improvement in this field of research was the development of nuclease resistant backbone structures, which – in addition to biological stability – can confer improved hybridization properties. When considering antisense activity, however, the study of the cellular delivery of the oligonucleotide (i.e. the uptake and intracellular distribution) is an equally important parameter and stability alone is insufficient if molecules remain extracellularly located.

As a result of studies of cellular uptake and localization of oligonucleotides using fluorescently or radiolabeled molecules, it is generally believed that the uptake of polyanionic oligomers is an inefficient process and the results are highly dependent on cell line, concentration of oligonucleotides and specific sequences [3]. At high concentrations oligonucleotides can be taken up by fluid phase pinocytosis whereas at low concentration (<1 μM) the uptake is thought to take place by receptor-mediated endocytotic processes [4].

To assist the transport of oligonucleotides across the cell membrane, cationic lipids can be used. These cationic lipids differ in cytotoxicity, serum sensitivity, and efficacy and results are cell-type dependent [4]. Generally, however, cationic lipids facilitate the uptake of oligonucleotides *in vitro* and may reduce the amount of the oligomer needed to obtain an antisense effect.

In the present study, we report on the first studies of the uptake and intracellular distribution of hexitol nucleic acids (HNA) in HeLa cells. HNA represent a class of synthetic oligonucleotides with a new backbone structure leading to strong hybridization with complementary RNA [5–7]. The oligomer has a similar constitution as DNA, except that the deoxyribose residue is replaced by an 1,5-anhydrohexitol moiety (Fig. 1). It can be considered as a DNA analogue with a methylene function inserted between the ring oxygen atom and the 1′-carbon atom. Due to the poor RNase H inducing activity of an RNA-HNA duplex, its potential antisense effect is dependent on cellular uptake and efficient steric hindrance of processing and translation of the target RNA in the appropriate cell compartment.

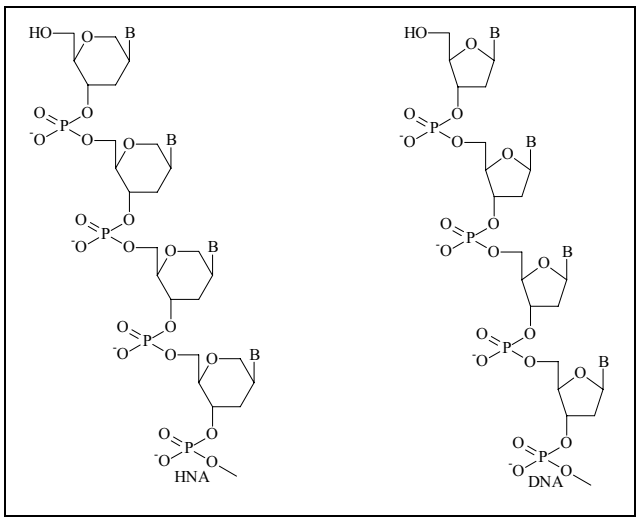

Fig. 1: Comparison of the structure of HNA and DNA

2. Investigations, results and discussion

It has been well established that the uptake of oligonucleotides within the cell can be assisted by using cationic lipid formulations [8]. In this study we assessed whether hexitol nucleic acids can be internalised by target cells in the presence or absence of lipid-based delivery reagents. In this series of experiments the human epithelial HeLa cell line was used as a model. In a preliminary experiment, the FITC-labelled oligos were incubated in the absence of carrier. Phosphorothioate-modified DNA (Fig. 1C) can be internalized more efficiently than HNA molecules (Fig. 1A) as measured by fluorescence detection. Treatment of cells with naked phosphorothioates resulted in detectable fluorescence in approximately 20% of cells (Fig. 1C).

The cellular uptake of fluorescently labelled HNA complexed with the transfection agents DOTAP, LipofectAmine, Superfect and CS075 were investigated. The cationic lipid formulation (1 or 2 μl) was complexed with 0.125, 0.625, 1.25 an 2.5 μM of the oligo and the mixture was incubated with HeLa cells in serum-free medium. The cells were assayed at 4 and 24 h by fluorescence microscopy for intracellular fluorescence. After 4 h there was no evidence of toxicity to the cells, and good intracellular fluorescence was seen with all reagents (Fig. 1B and 1D). Visual examinations led to the observations that the intensity of staining was oligo concentration-dependent over the range tested. The majority of cells demonstrated uptake of the oligo with a strong nuclear staining in some

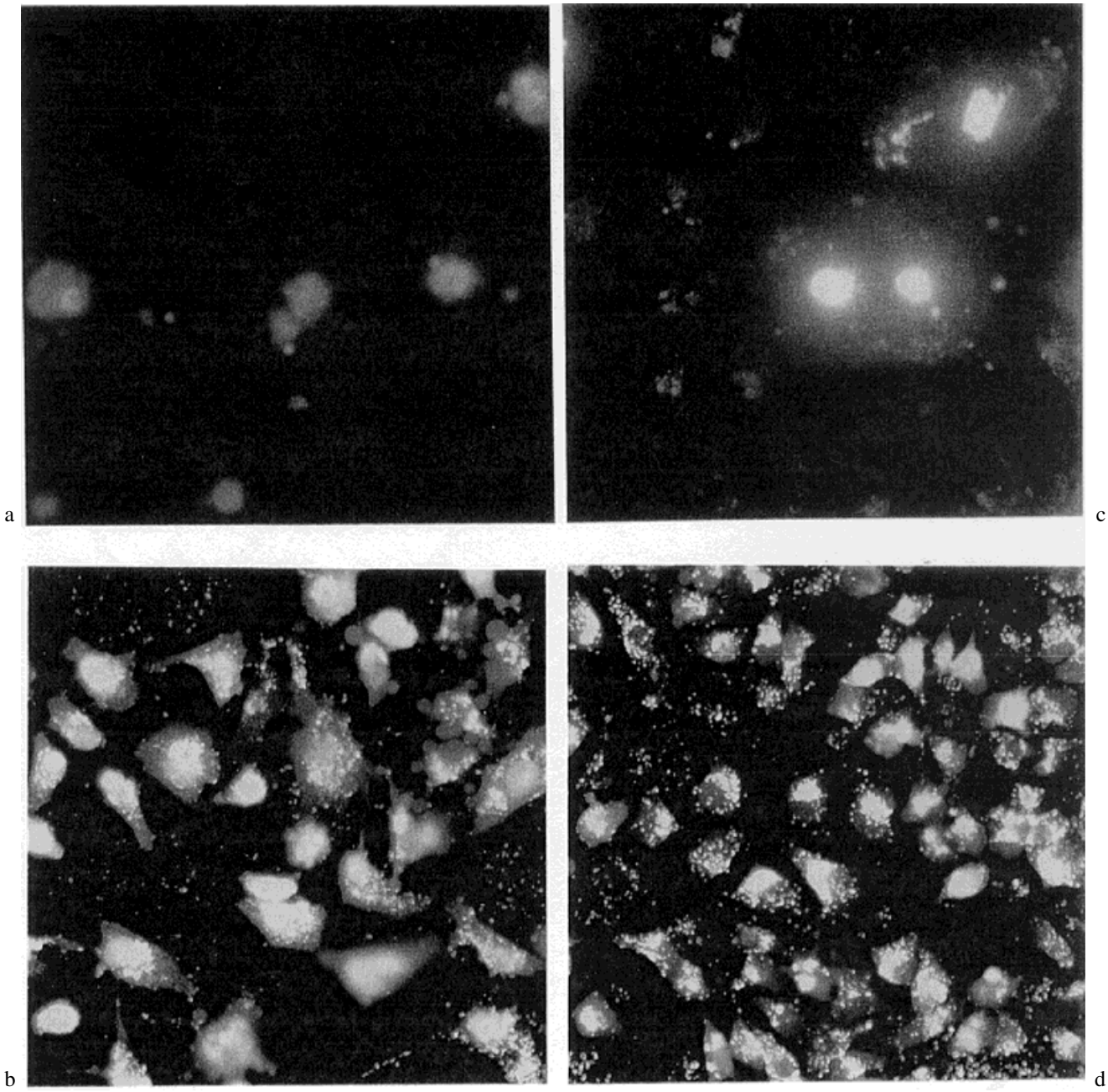

Fig. 2. Intracellular fluorescence in HeLa cells 24 hours after delivery of an anhydrohexitol (GC1187 E5) or phosphorothioate oligo, in the absence or presence of the lipid-based transfection reagent CS075. Cells were photographed on a Nikon microsope using a B2 filter (EX450-490, BA520).
a: (upper left) HNA without tranfection reagent
b: (lower left) HNA with transfection reagent (CS075)
c: (upper right) phosphorothioate without transfection reagent
d: (lower right) phosphorothioate with transfection reagent (CS075)

cells with all reagents tested. In addition to the nuclear signal, a faint, diffuse cytoplasmic signal could be seen in most cells. On occasion endosomal staining could be observed.

After 24 h the viability of the HeLa cells was diminished at higher concentrations of oligonucleotides for all cationic lipid formulations except for DOTAP. Generally, nuclear staining increased to 20–30% of the cells at 24 h. Whilst all reagents facilitated nuclear staining, CS075 produced the most consistant signal with 90% of cells observed showing strong endosomal staining and approximately 80% of cells exhibiting diffuse fluorescence throughout the nucleus. The toxicity of the CS075 was the lowest of all the reagents as shown by morphology of the cells following treatment.

The present investigations demonstrate for the first time conditions under which an 11-mer hexitol nucleic acid (HNA) was able to be delivered to the nucleus of HeLa cells. In the presence of a range of transfection reagents, the HNA molecules were detectable in a large proportion of the treated cells. The staining was endosomal, cytoplasmic and nuclear. The strong endosomal signal was comparable to that seen with the phosophorothioate oligo and is not necessarily a feature of the HNA chemistry. A HNA-specific feature was the lower efficiency of transfection in the absence of a transfection reagent. It is not clear

why this is the case, but a possible reason could be the inability of the HNA molecule to find the receptors responsible for internalization of other nucleic acids. It is obvious that small chemical modifications of a DNA structure can have a profound impact on cellular uptake. This simple method of delivery of the HNA paves the way to explore the potential of these highly stable molecules as gene suppression agents.

3. Experimental

The hexitol oligonucleotides were synthesized from the monomeric building blocks [6, 9] on an Applied Biosystems 392 DNA synthesizer on a 1 to 2 μmol scale. 1,*O*-Dimethoxytrityl-1,3-propanediol-functionalized LCAA-CPG was used as universal solid support, attaching a propanediol group at the 3′-end of the HNA oligonucleotide [10, 11]. The HNA oligomer was labelled at the 6′-end using fluorescein phosphoramidite as obtained from Eurogentec. The oligomers were deprotected, cleaved from the solid support and purified as described[6]. The HNA sequence used for studying the uptake: 6′-fluorescein-TAGCAGAGGAG-propanediol is an undecamer targeted to the 5'-cap sequence of ICAM-1 RNA. A reference 22-mer phosphorothioates of random sequence was prepared with a 5′-FITC label. A phosphorothioate oligonucleotide was used as reference because of its higher intra- and extracellular stability than a natural phosphodiester oligonucleotide. Three commerically available cationic lipid formulations were evaluated: DOTAP (Boehringer Mannheim), LipofectAmine, (Life Technologies) and Superfect (Qiagen). A fourth transfection reagent, CS075, composed of Tris conjugated to, respectively, a cationic head group and hydrophobic moieties, was also used [12].

The uptake of the oligonucleotides in the presence of transfection agents was studied as follows : HeLa cells (passage 4) were seeded the day before the experiments in 8-well chamber slides. The fluorescently labelled HNA was assayed at 4 different concentrations (2.5 μM, 1.25 μM, 0.625 μM and 0.125 μM). The cationic lipids were assayed at 1 and 2 μl per well (200 μl). The results were evaluated after 4 h and 24 h. The following protocol was used: a) the oligos were diluted to double-strength in 100 μl serum-free RPMI in 96-well plate; b) the cationic lipids were diluted (1 and 2 μl) in 100 μl serum-free RPMI in 96-well plate; c) the HNA oligomer and the cationic lipids were mixed in polystyrene 96-well plates and incubated at room temperature for 15 min; d) medium was removed from wells of HeLa cells; e) the HNA/cationic lipid mixtures were added dropwise to the HeLa cells which were seeded the previous day in 8-well chamber slides and incubated at 37 °C in 5% CO_2 atmosphere; f) at various time points the cells were washed in PBS, and the slides were mounted in 50% glycerol in PBS and observed on a Nikon microscope with a B2 EX450–490 excitation filter, BA520 barrier filter; g) cells were photographed with Kodak P1600 colour reversal slide film or Fujicolor 1600 film for colour prints. Carrier free experiments were completed as above except no carrier was added in a separate step (e).

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