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Synthesis and properties of oligonucleotides carrying isoquinoline imidazo[1,2-a]azine fluorescent units.

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TITLE RUNNING HEAD. Fluorescent labeling of oligonucleotides

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Abstract. Oligonucleotides carrying novel fluorescent compounds with a dipolar isoquinoline imidazo[1,2-a]azine core were prepared. Analysis of the melting curves demonstrates that DNA duplexes carrying these fluorescent labels at their ends have a slight increase on DNA duplex stability. The UV absorption and fluorescent properties of the oligonucleotide conjugates were analyzed. The fluorescent label is sensitive to duplex formation as cooperative melting curves are also observed at 366nm and fluorescence has a large increase upon denaturation. Cell-uptake studies allow observation of these fluorescently-labeled oligonucleotides internalized into HeLa cells.

KEYWORDS. Oligonucleotides, fluorescence, multicomponent reactions.

Introduction

There is a considerable interest in the development of new dyes and the introduction of these into biomolecules such as peptides, proteins and nucleic acids (*1*). Furthermore, the development of new methodologies for the selective and straightforward modification of biomolecules is having an increasing impact on biopharmaceuticals. When fluorescent molecules are attached to biomolecules that are not fluorescent, the resulting new entity acquires the fluorescent properties. Thus, fluorescent labels allow sensitive and selective detection of a specific component of complex biomolecular assemblies. Fluorescence-labeled oligonucleotides have large number of applications in several fields, such as biotechnology (*2*), cell biology (*3*), molecular biology (*4*), and biophysics (*5*). Therefore, the development of new fluorescent labels for oligonucleotides is of particular relevance (*6*, *7*).

Recently, we described the synthesis of a new class of fluorescent mesoionic acid fluorides using a multicomponent strategy. The reaction involves the participation of an azine, an isocyanide and a fluorinated anhydride and yields dipolar derivatives with an isoquinoline imidazo[1,2-a]azine core (see scheme 1) (*8*). These compounds are fluorescent, and when trifluoroacetic anhydride is used, they contain a reactive acid fluoride group that can be used to introduce the fluorescent label, by means of amide formation, into biomolecules that carry amino groups. The modular character of this

multicomponent reaction (MCR) (*9*) allows the convergent synthesis of a large variety of structural analogs in a single step. Thus both the fluorescent and biological properties of the dye can be optimized for specific applications. In this respect, the present study determines the features of the conjugation of these stable dipolar acyl fluorides with oligonucleotides, a fruitful strategy often used in peptide synthesis (*10*), which to our knowledge is unprecedented in the oligonucleotide field.

Here we report the conjugation of mesoionic acid fluorides **1a** and **1b** with oligonucleotides carrying alkyl amino groups (scheme 2) and demonstrate that fluorescence-labeled oligonucleotides can be prepared in good yields directly from the reaction of amino-oligonucleotides and mesoionic acid fluorides in aqueous media. Dipoles **1a** and **1b**, bearing respectively cyclohexyl and benzyl group as substituents at the imidazole nitrogen, were selected from a set of described analogues for the following reasons. First, they can be synthesized in multigram scale with a simple methodology and are well characterized (X-Ray, NMR and spectroscopy); also their substituents at the imidazole nitrogen (cyclohexyl and benzyl) featuring relevant differences in terms of shape, volume, polarity and conformational behavior, may confer to the corresponding bioconjugates distinct properties. Furthermore both residues are chemically stable, unlikely to suffer degradation in physiological media and, from an exploratory point of view, easy to replace by a wide number of analogues.

Our results indicate that the novel fluorescent oligonucleotides show potentially interesting properties for denaturation monitoring procedures as well as for cellular uptake studies.

Experimental Procedures

The phosphoramidites and ancillary reagents used during oligonucleotide synthesis were from Applied Biosystems (PE Biosystems Hispania S.A., Spain) and Link Technologies Ltd. (Scotland). The rest of the chemicals were purchased from Aldrich, Sigma or Fluka (Sigma-Aldrich Química S.A., Spain). 1-Cyclohexyl-3-(fluorocarbonyl)-2-oxo-2,3-dihydro-1*H*-imidazo[2,1-*a*]isoquinolin-4-ium-3-ide (**1a**), 1-benzyl-3-(fluorocarbonyl)-2-oxo-2,3-dihydro-1*H*-imidazo[2,1-*a*]isoquinolin-4-ium-3-ide (**1b**) 1-cyclohexyl-3-(4-methoxybenzylcarbamoyl)-2-oxo-2,3-dihydro-1H-imidazo[2,1-a]isoquinolin-4-ium-3-

ide (**1c**) and 1-cyclohexyl-3-(methoxycarbonyl)-2-oxo-2,3-dihydro-1H-imidazo[2,1-a]isoquinolin-4-ium-3-ide (**1d**) were prepared following literature procedures (*8*) (scheme 1).

Synthesis of oligonucleotides. Oligonucleotide sequences A: 5'-CCAATTGG-3', and B: 5'-TTCCGGAA-3' and C: 5'-CTCTCGCACCCATCTCTCTCCTTCT-3' were prepared on an automatic Applied Biosystems 3400 DNA synthesizer on 0.2- or 1-μmol scale using commercially available chemicals. The sequences were prepared with and without the amino group at the 3'-end. The synthesis was carried out using the standard phosphite triester methodology and a controlled-pore-glass support carrying the 2-(*N*-{[(9*H*-fluoren-9-yl)methoxy]carbonyl}-4-aminobutyl)propane-1,3-diol linker (*11*) (scheme 2) or in regular low-volume (LV200) polystyrene supports. The benzoyl (Bz) group was used to protect the amino group of dC and dA, and the isobutyryl (ibu) group to protect dG. Coupling yields were >95%. The last DMT group was removed at the end of the synthesis. The oligonucleotide was deprotected and cleaved from the support by treatment with aqueous concentrated ammonia for 12 h at 55ºC. Under these conditions, all protecting groups, including Fmoc were removed to yield unprotected 3'-amino-oligonucleotide. The mixture was filtered and the ammonia solution was concentrated to dryness. The residue was passed through a *Dowex 50x4* (Na^+ form) column to exchange ammonium ions for Na^+ ions. The solution was analyzed by HPLC and used directly in the dye conjugation. Analytical HPLC solutions: Solvent A: 5% acetonitrile (MeCN) in 100 mM triethylammonium acetate (pH=7) and solvent B: 70% MeCN in 100 mM triethylammonium acetate (pH=7). Column: X-Bridge ^{TM}OST C18 (2.5 μm 4,6x50 mm). Flow rate: 1 mL/min. Conditions for the analysis of octamers: 10 min linear gradient from 0-30% B at 60ºC; for the analysis of 25mer: 4 min linear gradient from 0-12% B, then 6 min linear gradient from 12-50% B at 60ºC). The desired oligonucleotides eluted at 2.5-3.5 min. Alternatively, oligonucleotides were purified by HPLC (see below).

Octamer sequence A (200 nmol scale): 33% yield (5 OD_{260}) t_R=2.6 min. Octamer sequence A 3'-NH_2 (1 μmol scale): 47% yield (36 OD_{260}), t_R=2.6 min. MALDI-TOF MS *m/z* (negative mode, THAP-CA, [M-

H]$^-$) expected for $C_{85}H_{106}N_{31}O_{50}P_8$ 2617.8, found 2618.7). Octamer sequence B (200 nmol scale): 39% yield (6 OD_{260}) t_R=3.2 min. Octamer Sequence B 3'-NH_2 (1 μmol scale): 54% yield (42 OD_{260}), t_R=3.2 min. MALDI-TOF MS *m/z* (negative mode, THAP-CA, [M-H]$^-$) calculated for $C_{85}H_{106}N_{31}O_{50}P_8$ 2617.8, found 2617.9). 25mer sequence C 3'-NH_2 (1 μmol scale): 58% yield 118 OD_{260}), t_R=4.0 min. MALDI-TOF MS *m/z* (negative mode, THAP-CA, [M-H]$^-$) expected for $C_{244}H_{301}N_{73}O_{159}P_{25}$ 7600, found 7603.6).

General protocol for conjugation. The amino-oligonucleotides were reacted with 1-cyclohexyl-3-(fluorocarbonyl)-2-oxo-2,3-dihydro-1*H*-imidazo[2,1-*a*]isoquinolin-4-ium-3-ide (**1a**) or 1-benzyl-3-(fluorocarbonyl)-2-oxo-2,3-dihydro-1*H*-imidazo[2,1-*a*]isoquinolin-4-ium-3-ide (**1b**) or with fluorescein isothiocyanate (**FITC**) (Isomer I, Sigma) as follows. Oligonucleotides (7 O.D.) were dissolved with 250 μL of an aqueous solution of $NaHCO_3$ 0.2M (pH=9). Then, 10 equiv of a solution of compound **1a** or **1b** or **FITC** in 0.25 ml of *N*,*N*-dimethylformamide (DMF) were added and the mixture was left to react for 8 h at room temperature. Then 100 μL of an aqueous solution of $NaHCO_3$ 0.2M (pH=9) and an additional 10 equiv of compound **1a** or **1b** in 100 μL of DMF were added and the mixture was left overnight at room temperature. The mixtures were concentrated to dryness, and the residues were dissolved in 1 ml of H_2O. The solutions were purified by *Sephadex G-25* (*NAP-10* columns). The fractions containing oligonucleotides were analyzed by HPLC as described above (Figure 1). Octamer **5** and 25mer **6** were used in the next step without further purification. Octamers **2-4** were purified by HPLC on a *Nucleosil* 120C18 (10μm, 200x10 mm) column. Flow rate: 3 mL/min. Conditions: 10 min linear gradient from 0-30% B, then a 10 min linear gradient from 30-100% B. The resulting products were desalted with *Sephadex G-25* (*NAP-5* Column). The coupling efficiency was determined by HPLC analyses (Octamer **2** 91%, Octamer **3** 91%, Octamer **4** 94%, 25mer **6** 85%, Octamer **5** (**FITC**) 94%). HPLC solutions as described above. Column: X-Bridge ^{TM}OST C18 (2.5 μm 4,6x50 mm). Flow rate: 1 mL/min. Conditions: 4 min linear gradient from 0-12% B, then a 1 min linear gradient from 12-40% B, then 5 min linear gradient from 40-45%B at 60ºC. The purified oligomers were analyzed by MS (MALDI-TOF) and UV-vis (Table 1).

Octamer **2**: 39% yield (2.7 OD_{260}), t_R=6.7 min., λ=260 and 347 nm, MALDI-TOF MS *m/z* negative mode, THAP-CA, $[M-H]^-$ calculated for $C_{103}H_{122}N_{33}O_{52}P_8$ 2910.1, found 2911.8.

Octamer **3**: 54% yield (3.8 OD_{260}), t_R=6.7 min., λ=260 and 346 nm, MALDI-TOF MS *m/z* negative mode, THAP-CA, $[M-H]^-$ calculated for $C_{103}H_{122}N_{33}O_{25}P_8$ 2910.1, found 2906.8.

Octamer **4**: 26% yield (1.8 OD_{260}), t_R=6.4 min., λ=260 and 345 nm, MALDI-TOF MS *m/z* negative mode, THAP-CA, $[M-H]^-$ calculated for $C_{104}H_{118}N_{33}O_{52}P_8$ 2918.3, found 2914.9.

Octamer **5**: 84% yield (5.9 OD_{260}), t_R=5.8 min., λ=260 and 493 nm, MALDI-TOF MS *m/z* negative mode, THAP-CA, $[M-H]^-$ calculated for $C_{106}H_{128}N_{32}O_{55}P_8S$ 3008.9, found 3004.0.

25mer **6** (from 30 OD_{260}): 71% yield (21.3 OD_{260}), t_R=8.3 min (4-min linear gradient from 0-12% B, then 6-min linear gradient from 12-50% B, at 60ºC), λ=268 and 348 nm; MALDI-TOF MS *m/z* negative mode, THAP-CA, $[M-H]^-$ expected 7892.3, found 7870.2.

Melting experiments. Melting experiments were performed in duplicate at concentrations ranging from 1.5-14 μM of duplex. Solutions of each oligonucleotide were mixed in a solution containing 1M NaCl and 100 mM sodium phosphate buffer pH=7. The DNA concentrations were determined by UV-absorbance measurements (260 nm) at 80ºC, using the ε_{260} values calculated by the nearest-neighbor method for the DNA coil state. For octamer **5** the ε_{260} value was calculated by the nearest-neighbor method as well and adding the contribution of fluorescein (the extinction coefficient of fluorescein (measured as FITC) at 260nm is around 13.7 $mM^{-1}cm^{-1}$). Samples were heated at 90ºC for 5 min, allowed to cool slowly to room temperature to induce annealing, and then kept overnight in a refrigerator (4ºC).

Melting curves were recorded by heating the samples with a temperature controller from 15 to 80ºC at a constant rate of 1ºC/min and monitoring the absorbance at 260 nm. During the experiment, when the temperature was below 25ºC, argon was flushed to prevent water condensation on cuvettes. Absorption

spectra and melting experiments (absorbance vs. temperature) were recorded in 1-cm-path-length cells. Melting curves were analyzed by computer-fitting the denaturation data, using Meltwin 3.5 software. Melting temperatures (Tm) decreased with the concentration *c*, as expected for a bimolecular reaction (*12*). The plot of 1/Tm *vs*. ln*c* was linear, giving a slope and a *y*-intercept, from which ΔH, ΔS and ΔG were obtained (Table 2).

Fluorescence analysis.

Fluorescence spectra were measured in solutions containing 1M NaCl and 100 mM sodium phosphate buffer (pH=7) at a concentration of 2.3 μM. Excitation was set at 345 nm for octamers **2-4** and 493 nm for octamer **5**. Fluorescence spectra were measured before (F_D) and after (F_{RC}) duplex denaturation to yield relative fluorescence intensity (F_{RC}/F_D)). Results are shown in Figure 2 and Table 4.

Flow cytometry experiments.

HT-29 cells were incubated for 8h with dipole **1c** at concentrations of 2 μM, 5 μM, 10 μM, 20 μM and 40μM in a low glucose DMEM (Biological Industries) medium in the absence of phenol red. A blank control was also prepared. The supernatant was separated and placed in falcon tubes. The cells were treated with trypsin and placed in the corresponding falcon tubes. Each tube was centrifuged for 4 min at 2000 RPM. The supernatant was removed and PBS (500 μL) was added to each tube. The samples were treated with a propidium iodide solution (5 μL of a 1 μg/mL solution) and analyzed with a Cell Lab Quanta SC cytometer (Beckman Coulter).

Confocal microscopy experiments

HT-29 cells were incubated with dipole **1c** and **1d** (20μM) for 2 h in a DMEM medium without phenol red. The living cells were treated with the plasmatic membrane marker WGA (Texas Red-x)-D (excitation at 595nm, emission 615nm). After 15 min the cells were analyzed *in vivo* with a Leica TCS SP2 confocal microscope (excitation at 350 nm, emission between 400 and 500 nm). In these

experiments, a blank control with the cells in the absence of dipole **1c** or its conjugate was analyzed. The intensity of the laser was adjusted in order to prevent detection of autofluorescent processes. Products **1c** and **1d** were detected in the cytoplasmatic region and they formed well defined aggregates (see Supporting Information). Using the same procedure, HeLa cells were incubated with **1c** and oligonucleotide **6** (20μM) for 4 h. While **1c** showed the same result as in the previous experiment, no fluorescence was detected using oligonucleotide **6**. In the presence of Lipofectamine^{TM} 2000 oligonucleotide **6** penetrated the cytoplasmatic region.

Results and Discussion.

Synthesis and thermodynamic properties of fluorescence labeled oligonucleotides

Small ligands can be incorporated into synthetic oligonucleotides at specific sites by preparing oligonucleotides carrying aliphatic amino groups and performing a conjugation reaction with the carboxylic derivatives of the ligands (*11*,*13*,*14*). This strategy was used to incorporate the acyl fluorides derivatives **1a** and **1b** into oligonucleotides (Scheme 2). Oligonucleotide sequences (Table 1) carrying an amino group at the 3'-end were assembled using a controlled pore glass support carrying the 2-(*N*-{[(9*H*-fluoren-9-yl)methoxy]carbonyl}-4-aminobutyl)propane-1,3-diol linker (*11*) and following standard protocols. The resulting oligonucleotides were used in the reaction with acyl fluorides (Scheme 1).

Amino-oligonucleotides were reacted with an excess of acyl fluorides **1a** and **1b** in aqueous dimethylformamide (DMF) mixtures at pH 9 and room temperature. In these mild conditions the desired fluorescent oligonucleotides **2-4, 6** were produced in good yields as shown by the presence of new products with higher retention time, which were characterized by mass spectrometry and UV spectra (Figure 1 and Table 1). In addition we have prepared the fluoresceine-labelled octamer **5** by reaction of the appropriate amino-octamer with fluoresceine isothiocyanate (**FITC**).

The hybridization properties of the oligonucleotides carrying the isoquinoline imidazo[1,2-a]azine group were measured spectrophotometrically on the self-complementary octamer duplexes **2-4**. Unmodified octamers were included for comparison purposes (Table 2). The duplex formed by sequence **A** which carried two molecules of compound **1a** (octamer **2**), melted at 41.1 ºC (ΔG 20.6 kcal/ mol), while the unmodified duplex melted at 33.3 ºC (ΔG 18.0 kcal/ mol). This observation implies an increase in duplex stability of 3.8 ºC (ΔΔG 1.3 kcal/ mol) per fluorescent molecule. In contrast, the duplex formed by sequence **A**, carrying two molecules of compound **1b** (octamer **4**), melted at 34 ºC (ΔG 17.7 kcal/ mol), thereby indicating a small destabilization of the duplex. This effect should reflect the previously mentioned differences between the substituents on the imidazole nitrogen of the dipoles, presumably arising from the distinct interactions at the conformational level of the cyclohexyl and benzyl groups with the oligonucleotide backbone. The stabilizing properties of compound **1a** were slightly increased in octamer **3** (ΔΔG 2.0 kcal/ mol), thereby indicating that the stabilizing properties of this compound are not dependent on the DNA sequence at the end as octamer **3** has A.T base pairs while octamer **2** has G.C base pairs.

Preliminary conformational studies of the linkers containing cyclohexyl or benzyl substituents by means of computer-based molecular modeling (Spartan'04 and Hyperchem'08; molecular mechanics conformational search and geometry optimization) suggest that in the lower energy conformations, the isoquinoline imidazo[1,2-a]azine core containing a cyclohexyl substituent stacks over the terminal guanine nucleobase, at a distance of approximately 3 to 4 Å, whereas the isoquinoline imidazo[1,2-a]azine core containing a benzyl substituent does not interact significantly with the terminal guanine (see Supplementary Figure S1). These studies were carried out by keeping frozen the duplex of DNA and by allowing the linker to move freely during the calculations. In order to explore the rotational freedom of the cyclohexyl or benzyl-fluorescent isoquinoline imidazo[1,2-a]azine in more detail and to determine the dynamic behavior of the conjugates, Molecular Dynamics simulations and NMR studies

on these modified duplexes would be necessary. A comprehensive study is currently under way in our laboratory.

UV spectra of the modified oligonucleotides **2-4** at 20ºC (duplex) and 70ºC obtained in a solution containing 1M NaCl and 100 mM sodium phosphate buffer of pH=7 at a concentration of 6.8 μM are shown in Figure 2a. The maximum caused by the isoquinoline derivative shows a slight shift depending on the temperature. The hyperchromism in the absorption spectra at 70ºC and the bathochromical shift indicated that the molecule attached to the oligonucleotide is sensitive to the presence of a duplex or a random coil form. The UV spectra indicated a change in hyperchromicity between 340 and 380 nm, the difference being maximum around 366 nm, so melting curves were recorded at 366 nm. Melting curves were recorded simultaneously at 260 nm and 366 nm for comparison purposes (Figure 3). Melting temperatures at these two wavelengths were similar, although at 366 nm the duplex showed a lower Tm (see for example compound **2** T_m: 42.9ºC at 366 nm and 43.2ºC at 260 nm, Table 3). This indicates that base pair dissociation observed at end of the oligonucleotide (measured at 366 nm where the fluorescent tag is located) happens at a slightly lower temperature of the global melting of the duplex (measured at 260 nm). A similar behaviour has been described for fluorescent compounds attached in the middle a DNA duplex (*15*, *16*). The fluoresceine octamer **5** had a melting temperature of the same range than octamers carrying the isoquinoline imidazo[1,2-a]azine core with the cyclohexyl group (**1a**) **2** and **3** (Table 3). Surprisingly, at 493 nm it had a slightly higher melting temperature (Tm 44.8 ºC at 493 nm, Tm 44.2ºC at 260 nm).

Next we studied the effects of duplex denaturation in fluorescence. Fluorescent spectra of self-complementary duplex **2** in 1M NaCl and 100 mM sodium phosphate buffer pH=7 at a concentration of 2.3 μM at different temperatures were recorded. Figure 2b shows the fluorescent spectra at 27 ºC and 53 ºC. Excitation was set at 345 nm Absorption maxima of oligonucleotides **2-4** at high and low temperatures are summarized in Table 4. A strong increase in fluorescence was observed upon duplex denaturation. These results and the slight increase in T_m indicates that the fluorescent label interacts

with the DNA duplex most probably as an end cap and the complex has a lower florescence. Upon duplex denaturation, the fluorescent label changes the environment duplicating the fluorescence emission. These effects were more intense in octamers carrying compound **1a** (oligonucleotides **2** and **3**) than those carrying compound **1b** (oligonucleotide **4**). This effect may be a consequence of the conformational preference of the isoquinoline imidazo[1,2-a]azine core containing a cyclohexyl substituent to stack over the terminal nucleobase described above. The fluoresceine octamer **5** does not increase the fluorescence upon heating, but, on the contrary, a clear decrease on the fluorescence properties is observed (Table 4).

In summary, dipolar isoquinoline derivatives **1a-b** readily prepared by MCR are efficient and inexpensive reagents for introducing of fluorescent labels into synthetic oligonucleotides. Reagent **1a** is particularly useful as it produces fluorescence-labeled oligonucleotides that are sensitive to hybridization and have duplex-stabilizing properties. Next we studied the capacity of fluorescence-labeled oligonucleotides to monitor cellular uptake.

Cell uptake experiments.

A 25 mer oligonucleotide sequence with an amino group at the 3'-end (Table1) was also prepared. This sequence corresponds to an antisense oligonucleotide known as GEM91 (*17*), which is complementary to the initiation codon of gag gene of HIV-1 RNA. Reaction of 3'-amino GEM91 with **1a** yielded the expected fluorescence-labeled oligonucleotide (**6)** in excellent yields (85% conversion judged by HPLC, 71% isolated yield). The resulting product was used in the transfection assays without further purification.

We first performed flow cytometry experiments with the model isoquinoline compound **1c** in order to assess the potential toxicity of the dye. HT-29 cells were incubated for 8 h with dipole **1c** and analyzed by flow cytometry. The percentage of living or dead cells remained almost constant at all concentrations of **1c**, and was very similar to the control value (see Supplementary Material). These results support the notion that this compound has a low toxicity profile.

We then performed confocal microscopy experiments to analyze the distribution of the compound **1c** inside the cells. Compound **1c** was detected in the cytoplasmatic region and formed well defined aggregates (see Supplementary Material). The analogous methyl ester dipole **1d** (*8*) was analyzed under the same conditions and showed a similar distribution pattern (see Supplementary Material).

We then performed further confocal microscopy experiments to optimize the conditions in order to evaluate the capacity of conjugate **6** to penetrate HeLa cells in the presence (or absence) of Lipofectamine^{TM} 2000. When cells were incubated with conjugate **6** (20μM) for 4 h, no fluorescence was detected, whereas the same product in the presence of Lipofectamine^{TM} 2000 penetrated the cytoplasmatic region. Longer incubation times (22 h) with conjugate **6 (**20μM) in the presence of Lipofectamine^{TM} 2000, induced strong cell toxicity and no living cells were found. This result may reflect the toxicity of Lipofectamine under these conditions.

Conclusions

Here we describe the use of unexpensive reagents in the synthesis of oligonucleotides carrying highly sensitive fluorophores. The acid fluoride derivative of the fluorophore required for the preparation of the fluorescent-labeled oligonucleotides was synthesized in high yield in a one-pot reaction using an efficient MCR which has been described previously (*8*). The acyl fluorides reacted efficiently in aqueous buffers at pH 9 with amino-oligonucleotides to yield the desired conjugates. The resulting oligonucleotides show intense fluorescent properties in presence of ions, have slightly improved hybridization properties and are sensitive to denaturation. Flow cytometry and confocal microscopy experiments showed a low toxicity profile and good cell membrane permeability respectively for dyes **1c** and **1d**. Oligonucleotide **6** had the capacity to penetrate cells in presence of Lipofectamine^{TM} 2000 transfection reagent.

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Supporting Information Available. Structure of the most energetically stable conformations of **1a** and **1b** conjugates by molecular mechanics (MM) conformational searches and geometry optimization. Flow cytometry experiments with dipolar amide **1c**. Confocal microscopy images from cell uptake experiments. Mass spectrometry, HPLC analysis and melting curves of purified oligonucleotides. This material is available free of charge via the internet at http://pubs.acs.org

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TABLES.

Table 1. Mass spectra and UV maxima of fluorescent oligonucleotides prepared in this study.

#	Sequence (5'-3')	MS (expected)	MS (found)	UV_{max}, nm
2	CCAATTGG-NHCO-**1a**	2910.1	2911.8	260, 347
3	TTCCGGAA- NHCO-**1a**	2910.1	2906.8	260, 346
4	CCAATTGG-NHCO-**1b**	2918.3	2914.9	260, 345
5	TTCCGGAA-NHCO-**FITC**	3008.9	3004.4	260, 493
6	CTCTCGCACCCATCTCTCTCCTTCT NHCO-**1a**	7892.3	7870.2	267, 348

Table 2. Thermodynamic parameters for duplex-to-random-coil transitions[*] (1M NaCl and 100 mM sodium phosphate buffer at pH=7).

#	Duplex	Tm [ºC][**]	Δ*H* [Kcal/mol]	Δ*S* [Kcal/mol.K]	Δ*G* [Kcal/mol]	ΔΔ*G*[***] [Kcal/mol]
	$^{5'}$CCAATTGG$^{3'}$	33.3	-119.5	-340.5	-18.0	0
2	$^{5'}$CCAATGG-**1a**$^{3'}$	41.1	-116.8	-322.5	-20.6	1.3
4	$^{5'}$CCAATGG-**1b**$^{3'}$	34.0	-103.7	-288.3	-17.7	-0.15
	$^{5'}$TTCCGGAA$^{3'}$	31.8	-107.5	-303.0	-17.2	0
3	$^{5'}$TTCCGGAA-**1a**$^{3'}$	44.2	-107.0	-287.8	-21.2	2

(*) Δ*G* is calculated at 25ºC, with the assumption that Δ*H* and Δ*S* do not depend on temperature; analysis was carried out with melting temperatures obtained from denaturation curves. (**) Tm calculated at 4μM duplex concentration. (***)ΔG (modified octamer)-ΔG (unmodified octamer) / 2 fluorescent molecules.

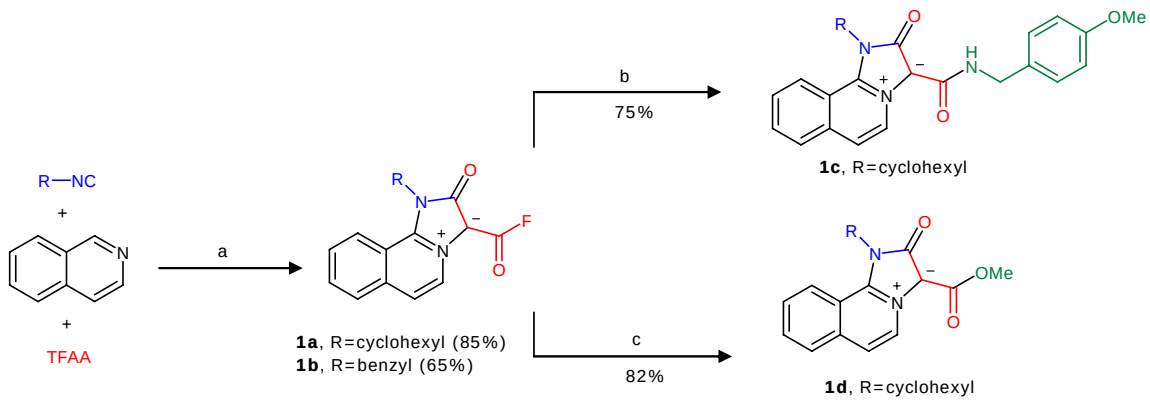
Table 3. Melting temperatures of modified and unmodified duplex at different λ.

#	Oligonucleotide	Abs. (260 nm, 70ºC)	Tm (ºC) (260 nm)	Tm (ºC) (λ)(*)
2	5'CCAATGG-**1a**3'	0.59	43.2	42.9
3	5'TTCCGGAA-**1a**3'	0.48	45.9	35.4
4	5'CCAATGG-**1b**3'	0.61	37.2	36.8
5	5' TTCCGGAA-**FITC**3'	0.63	44.2	44.8

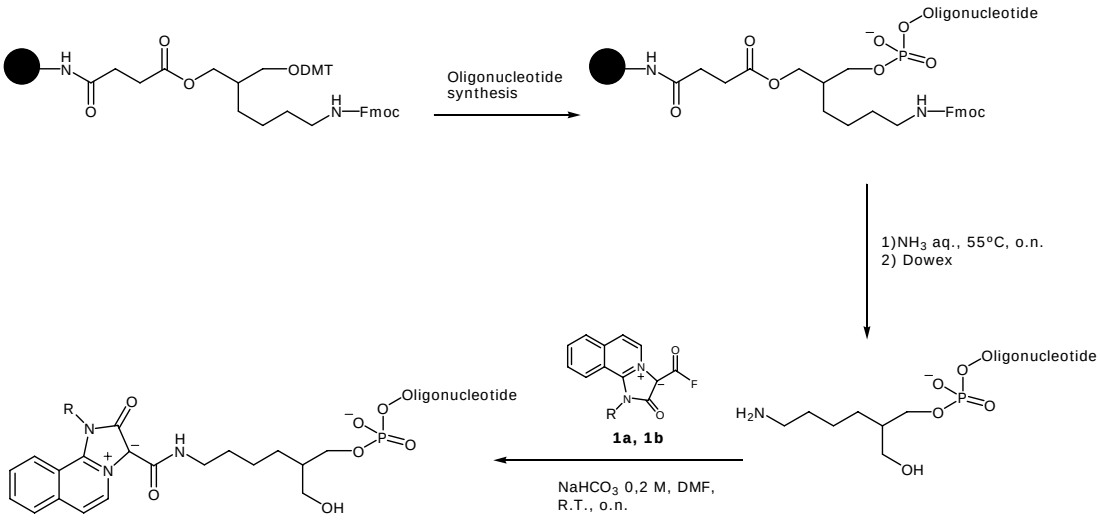
(*) Melting experiments performed at 366 nm for **2** and **4**, 364 nm for **3** and 493 nm for **5**

Table 4. Fluorescence spectral data for the modified oligonucleotides before and after duplex denaturation.

	Temperature (ºC)	Emission maximum (nm)	Fluorescence intensity (I)	F_{RC}/F_D	ΔI (%)
2	27	426	184	2.02	+102
	53	429	371		
3	24	427	322	1.74	+74
	51	430	560		
4	17	418	312	1.34	+34
	39	423	418		
5	20	522	117	0.82	-18
	60	520	96		



Scheme 1. 3-Component reaction of isoquinoline and isocyanides in the presence of trifluoroacetic anhydride and subsequent reaction of the resulting acyl fluoride with *p*-methoxybenzylamine or methanol. Reagents and conditions: a) CH_2Cl_2, 14 h, r.t., b) *p*-methoxybenzylamine, TEA, CH_2Cl_2 anh., r.t., overnight c) MeOH, TEA, reflux.



Scheme 2. Synthesis of oligonucleotides carrying a derivative of isoquinoline at the 3'-end.

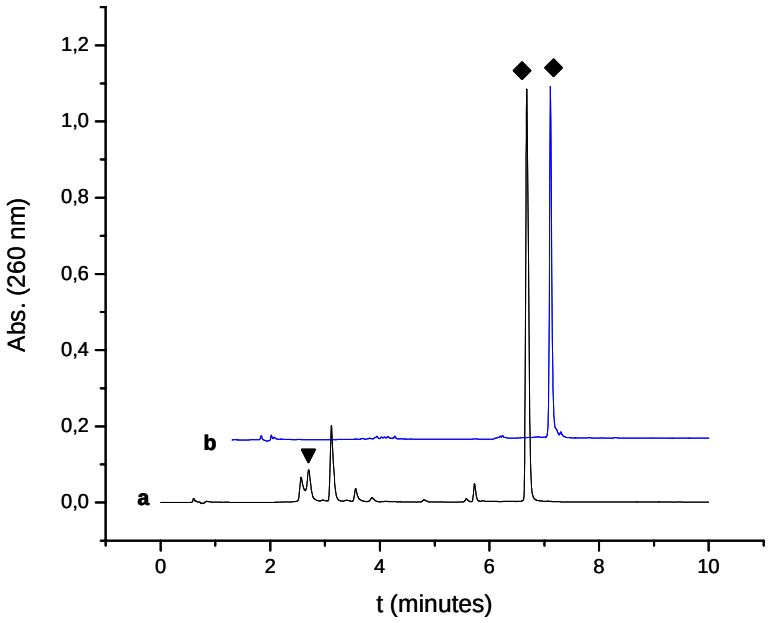


Figure 1. HPLC profiles of a) the mixture obtained after the reaction with compound **1a** and amino-oligonucleotide sequence A to yield compound **2** (conversion 91%) and b) purified compound **2**. HPLC conditions: 4 min linear gradient from 0-12% B, then 6 min linear gradient from 12-50% B at 60ºC. The desired oligonucleotide (compound **2**) eluted at 8.7 min (▼ stands for amino-oligonucleotide sequence A and ♦ stands for oligomer 2).

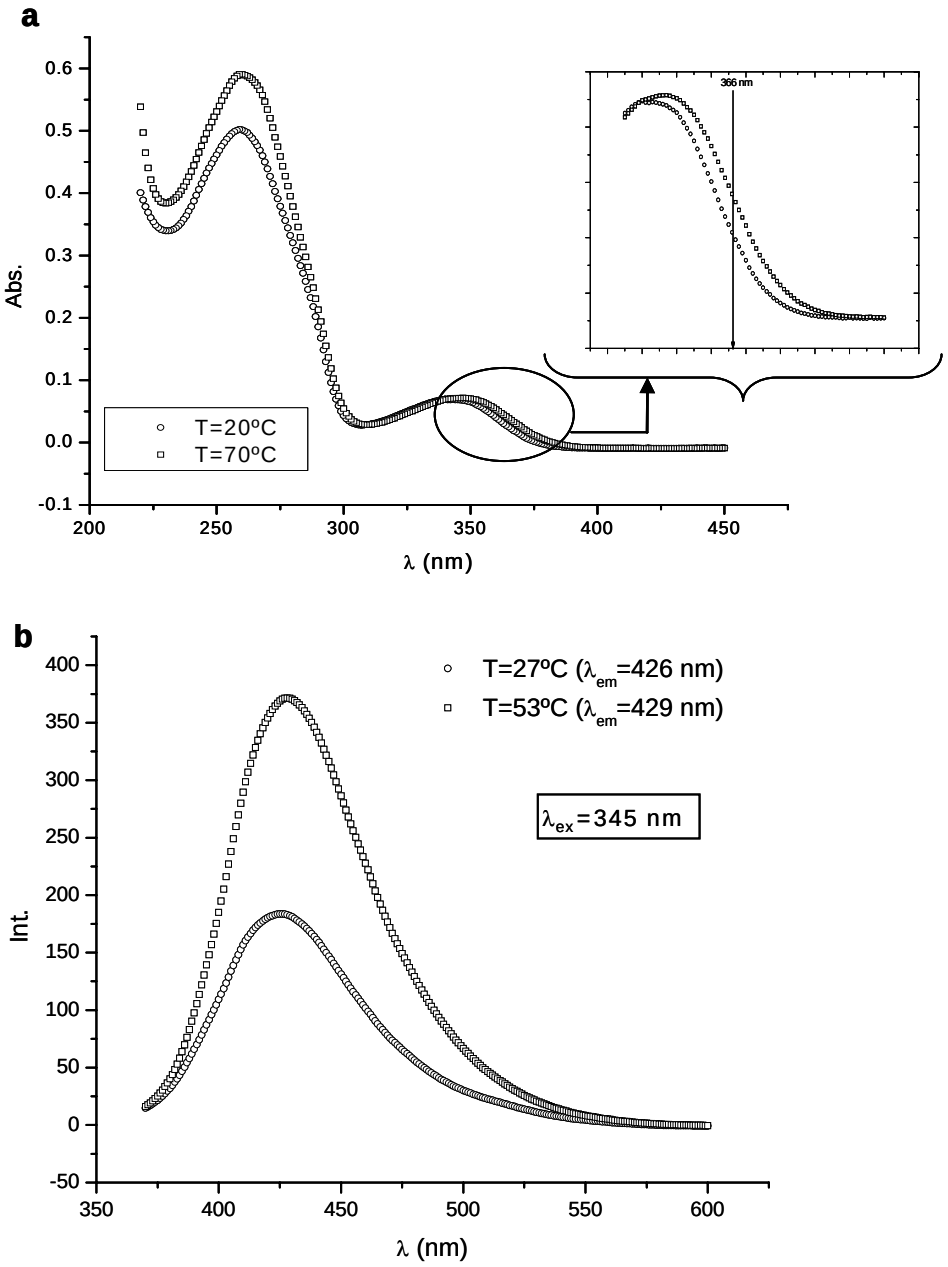


Figure 2. a) UV and b) fluorescence spectra of oligonucleotide **2**.

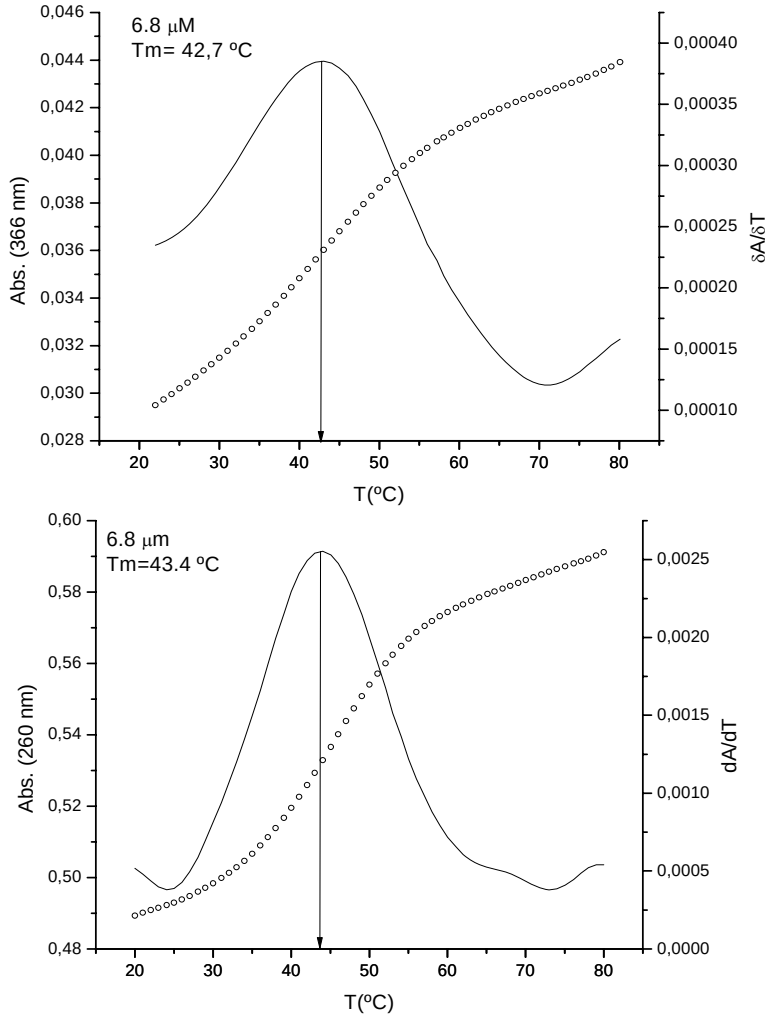
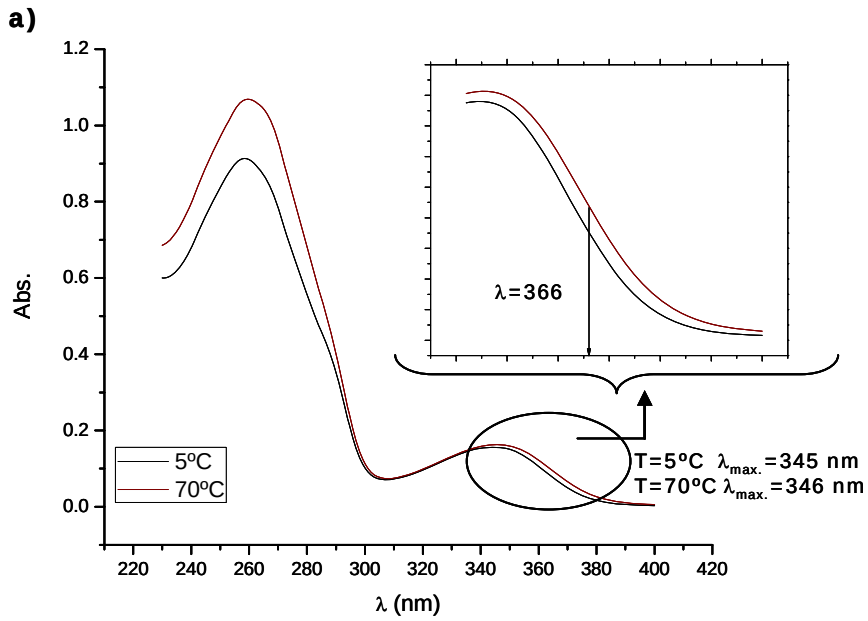


Figure 3. Melting curves of self-complementary oligonucleotide **2** measured at 260 nm and 366 nm.

SYNOPSIS



Supporting data

Synthesis and properties of oligonucleotides carrying isoquinoline imidazo[1,2-a]azine fluorescent units.

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SUPPORTING INFORMATION:

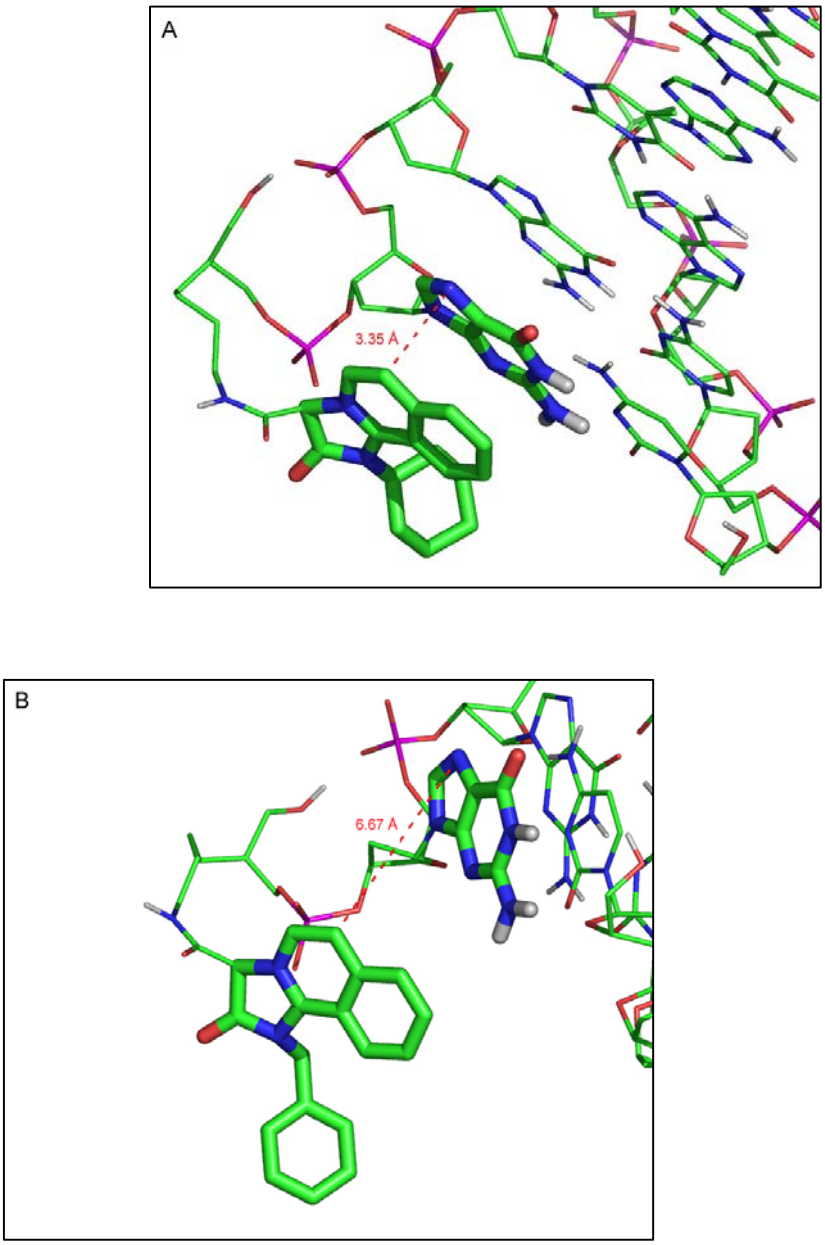

Figure S1. Most energetically stable conformations of **1a** and **1b** conjugates (panels A and B, respectively). The terminal guanine nucleobase and the triazine core are shown as thick lines. Distances between guanine and the isoquinoline imidazo[1,2-a]azine core are indicated. The figures were prepared with PyMOL (http://pymol.sourceforge.net/). Molecular mechanics (MM) conformational searches and geometry optimization of the most energetically stable conformers were carried out with the Amber force field by using the softwares Spartan'04 from Wavefunction and HyperChem'08 and by using water as a solvent. The duplex of DNA was kept frozen and the linker was allowed to move freely during the calculations.

Flow cytometry experimental data:

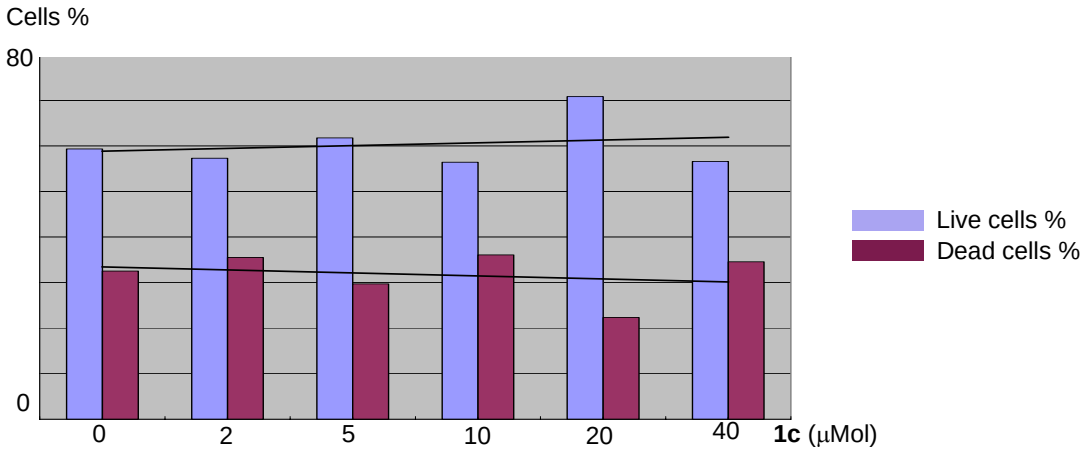

Figure S2: Evaluation of the cytotoxicity profile of **1c** by flow cytometry on HT-29 cells. Incubation time 8h.

Confocal microscopy images:

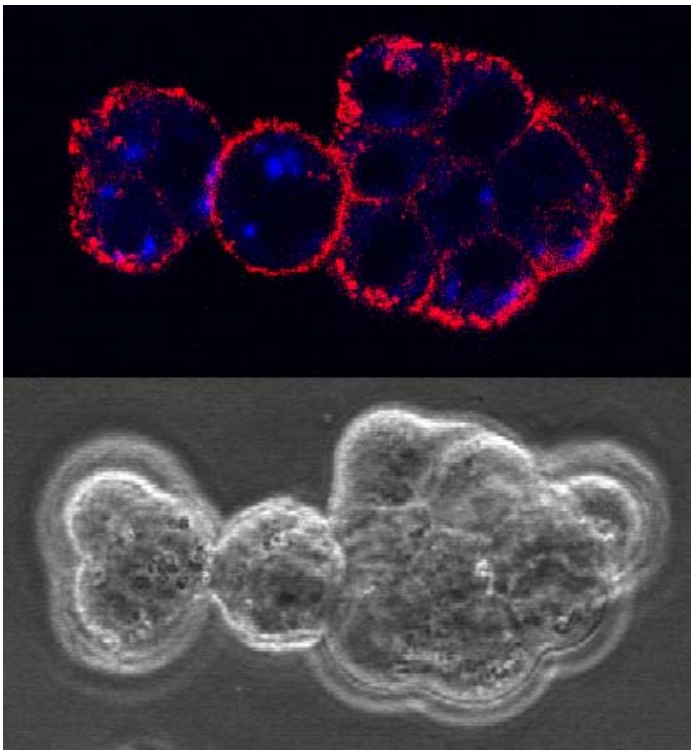

Figure S3: **1c** [20 μM] in HT-29 cells (2h incubation)

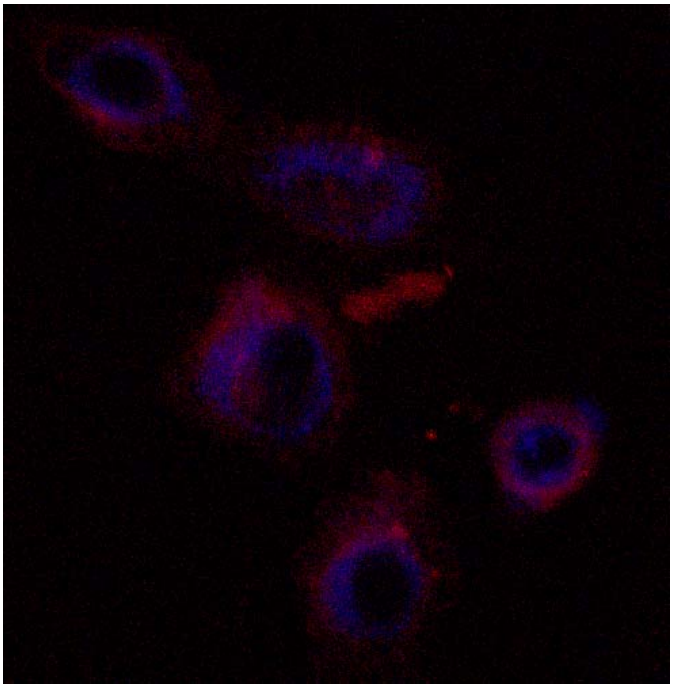

Figure S4.: **1c** [20 μM] in Hela cells (4h incubation)

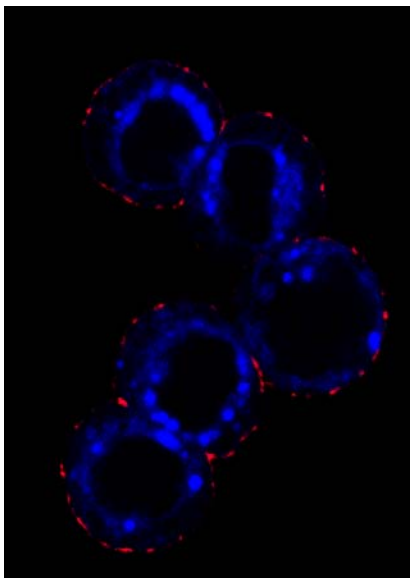

Figure S5: **1d** [20 μM] in HT-29 cells (2h incubation)

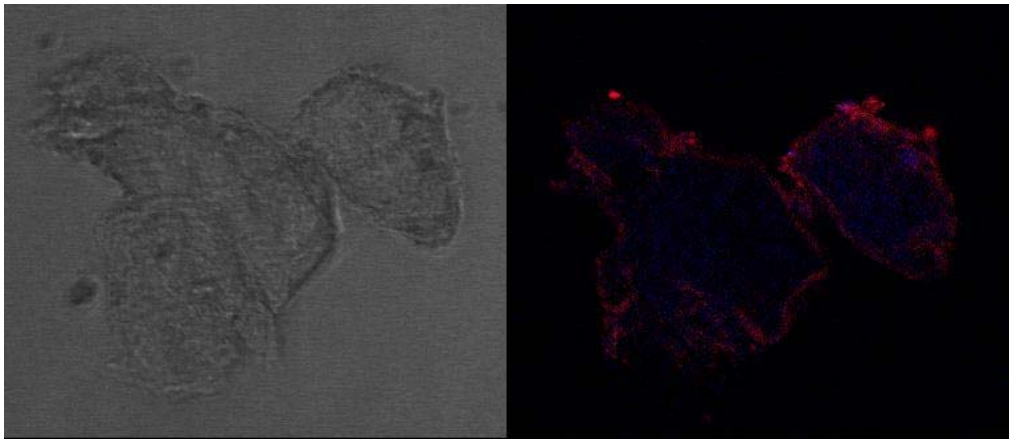

Figure S6.: Oligonucleotide **6** [20 μM] with Lipofectamine in Hela cells (4h incubation)

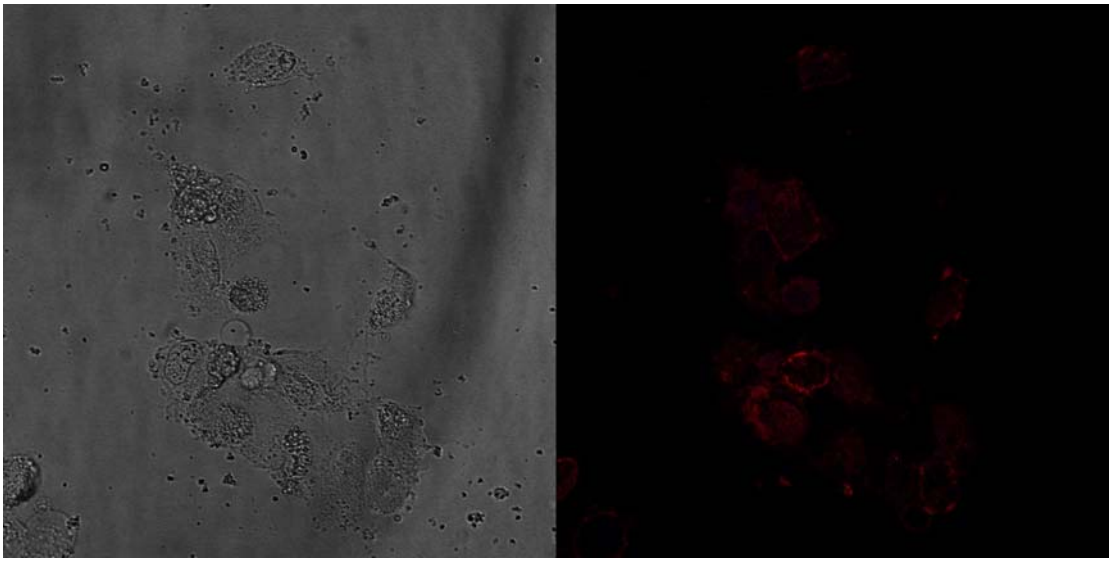

Figure S7. Oligonucleotide **6** [20 mM] with Lipofectamine in Hela cells (22 h incubation).