Delivery of Nucleic Acid Sequences in Mammalian Cells Mediated by Phosphorothioate DNA or RNA Transporter Elements

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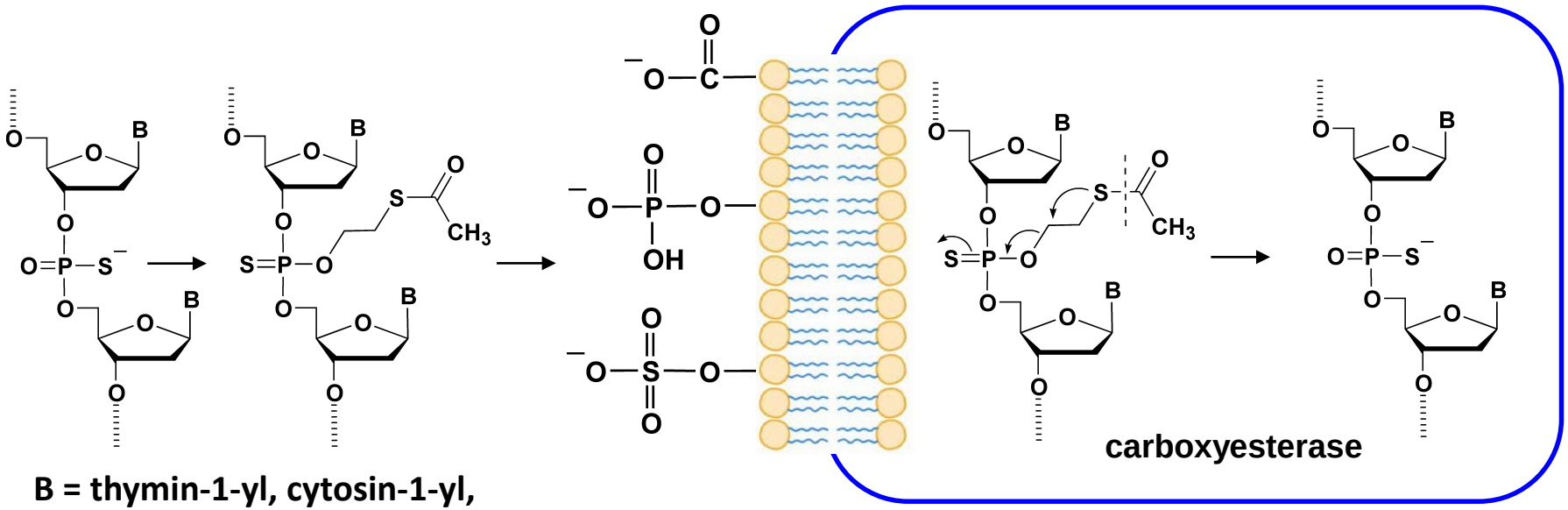
The Oligonucleotide Prodrug Approach to Cellular Delivery of Antisense DNA Oligonucleotides

Tosquellas, G.; Alvarez, K.; Dell'Aquila, C.; Morvan, F.; Vasseur, J. –J.; Imbach, J. –L.; Rayner, B. *Nucleic Acids Res.* 1998, *26*, 2069

Bologna, J.-C.; Vivès, E.; Imbach, J. –L.; Morvan, F.
Antisense Nucleic Acid Drug Dev. 2002, *12*, 33

The main objective of the prodrug approach was to provide lipophilicity and resistance to ubiquitous nucleases to therapeutic oligonucleotides while improving cellular uptake



B = thymin-1-yl, cytosin-1-yl, adenin-9-yl or guanin-9-yl

Limitations of this prodrug approach:

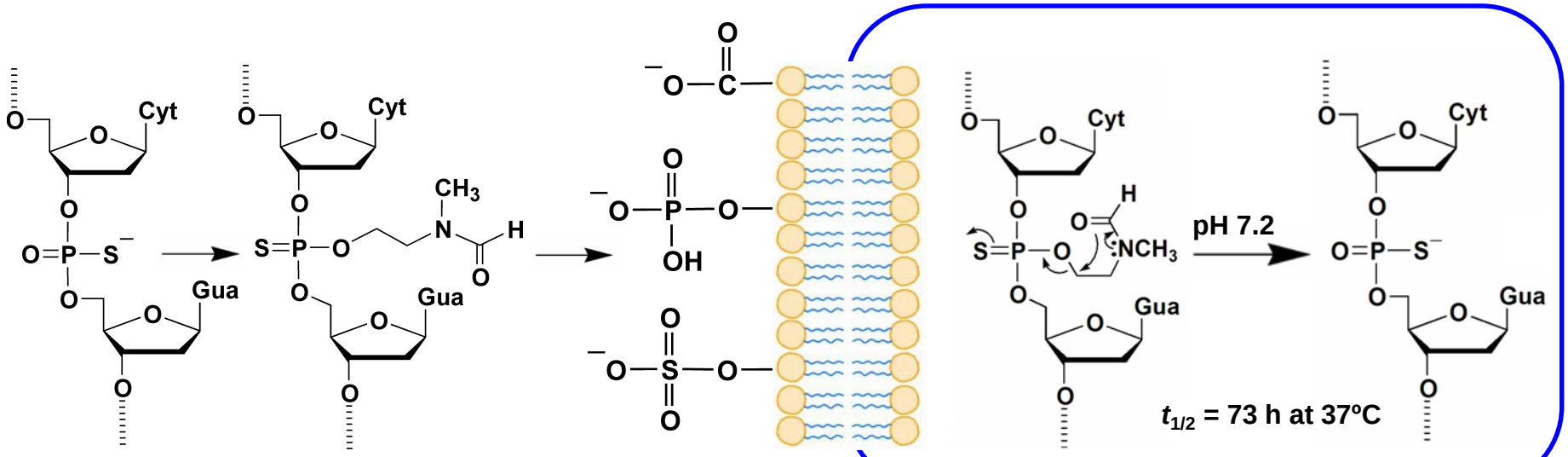
- Requires intracellular carboxyesterase(s)
- A more complex protection/deprotection strategy is needed for the chemical synthesis of these types of oligonucleotide prodrugs

Ferreira, F.; Vasseur, J. -J.; Morvan, F. *Tetrahedron Lett.* 2004, 45, 6287

Development of Thermolytic Oligonucleotide Prodrugs

Grajkowski, A.; Wilk, A.; Chmielewski, M. K.; Phillips, L. R.; Beaucage, S. L. *Org. Lett.* 2001, *3*, 1287.

Grajkowski, A.; Pedras-Vasconcelos, J.; Wang, V.; Ausín, C.; Hess,S.; Verthelyi, D.; Beaucage, S. L. *Nucleic Acids Res.* 2005, *33*, 3550.



CpG ODN 1555 **CpG ODN fma1555 prodrug**

Cyt, cytosin-1-yl
Gua, guanin-9-yl

Bioactive d(GCTAGACGTTAGCGT) [CpG ODN 1555] in mice

+ESI-TOF MS: calcd for $C_{147}H_{185}N_{57}O_{75}P_{14}S_{14}$ $[M + 14H]^+$ 4832, found 4832

Bioactive d($G_{fps}C_{fps}T_{fps}A_{fps}G_{fps}A_{fps}C_{fps}G_{fps}T_{fps}T_{fps}A_{fps}G_{fps}C_{fps}G_{fps}T$) [CpG ODN fma1555] in mice

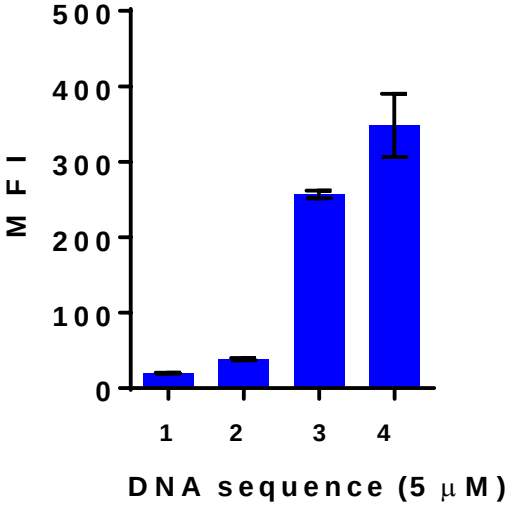
fps, 2-(*N*-formyl-*N*-methylamino)ethyl phosphorothioate triester

+ESI-TOF MS: calcd for $C_{203}H_{283}N_{71}O_{89}P_{14}S_{14}$ $[M]^+$ 6024, found 6023

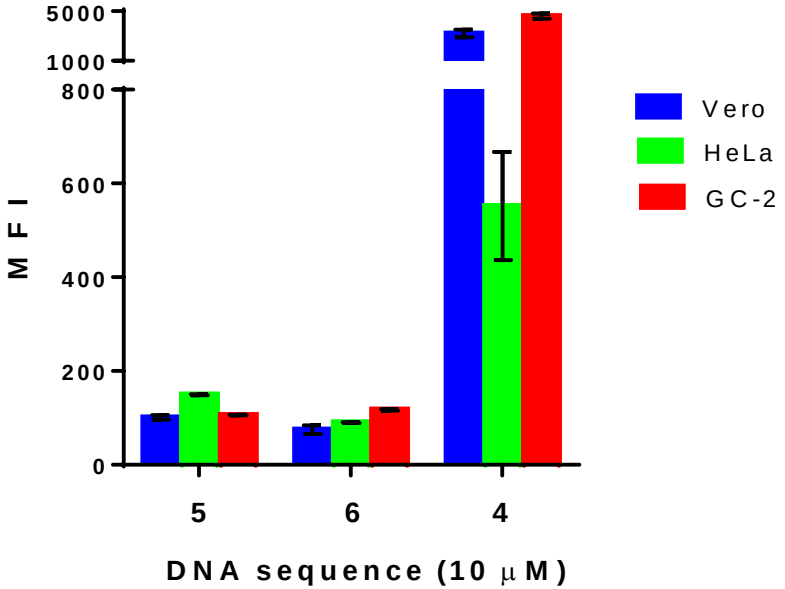
Internalization of Thermolytic Oligonucleotide Prodrugs in Mammalian Cells

Jain, H.V.; Takeda, K.; Tami, C.; Verthelyi, D.; Beaucage, S.L.
Bioorg. Med. Chem. 2013, 21, 6224

Comparative FACS analysis of the uptake of 5'-fluoresceinated phosphorothioate DNA sequences in Vero, HeLa and GC-2 cells



3 h at 37 °C



18 h at 37 °C

1 $Fl_{ps}G_{ps}C_{ps}T_{ps}A_{ps}G_{ps}A_{ps}C_{ps}G_{ps}T_{ps}T_{ps}A_{ps}G_{ps}C_{ps}G_{ps}T$ (CpG ODN 1555)

2 $Fl_{ps}G_{fps}C_{fps}T_{fps}A_{fps}G_{fps}A_{fps}C_{fps}G_{fps}T_{fps}T_{fps}A_{fps}G_{fps}C_{fps}G_{fps}T$ (CpG ODN fma1555)

3 $Fl_{ps}G_{+ps}C_{+ps}T_{fps}A_{fps}G_{fps}A_{fps}C_{fps}G_{fps}T_{fps}T_{fps}A_{fps}G_{fps}C_{+ps}G_{+ps}T$

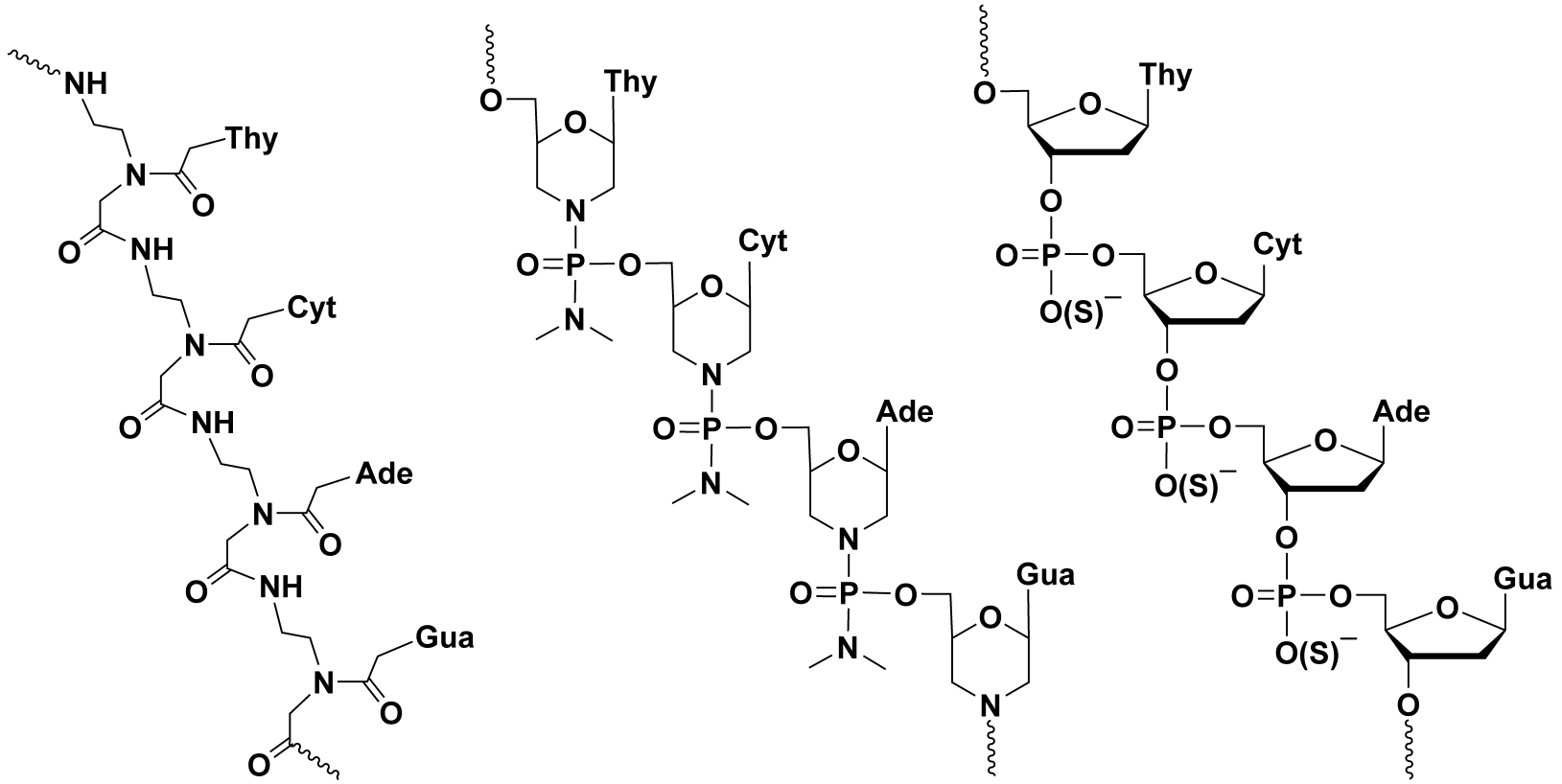
4 $Fl_{ps}(T_{+ps})_2(T_{fps})_{10}(T_{+ps})_2T$

5 $Fl_{ps}(T_{ps})_{14}T$

6 $Fl_{ps}(T_{fps})_{14}T$

Fl, fluorescein; MFI, mean fluorescence intensity ;ps, phosphorothioate diester; fps, 2-(*N*-formyl-*N*-methylamino)ethyl phosphorothioate triester; +ps, 3-(*N*,*N*-dimethylamino)propyl phosphorothioate triester

Peptide Nucleic Acid (PNA) and Phosphorodiamidate Morpholino (PMO) Sequences as Potential Nucleic Acid-Based Drugs



PNA **PMO** **DNA**

Thy, thymin-1-yl; Cyt, cytosin-1-yl; Ade, adenin-9-yl; Gua, guanin-9-yl

- Similar to thermolytic oligonucleotide prodrugs, PNA and PMO sequences are nuclease-resistant, RNase-H incompetent uncharged nucleic acid analogues and exhibit poor cellular uptake

- Although cationic lipids are efficient carriers for in vitro cellular delivery of negatively charged DNA/RNA sequences, these carriers cannot be successfully used for cellular internalization of uncharged PNA or PMO sequences.

- Similar to thermolytic oligonucleotide prodrugs, PNA and PMO sequences require to be positively charged for cellular internalization

- Conjugation of cationic cell-penetrating peptides (CPP) to PNAs or PMOs improved the cellular uptake of these oligomers and led to pre-mRNA splicing correction activities in mammalian cells and animal models.
- However, the preparation and purification of CPP-conjugates is tedious and labor intensive.
- CPP-PNA or -PMO conjugates are sensitive to intracellular peptidases (Youngblood et al., 2007) and prone to induce undesirable toxicity (Wu et al., 2012).
- There must be a simpler approach to the delivery of PNAs and PMOs in mammalian cells!

Youngblood, D.S., Hatlevig, S.A., Hassinger, J.N., Iversen, P.L., Moulton H.M. *Bioconjugate Chem.* 2007, 18, 50.

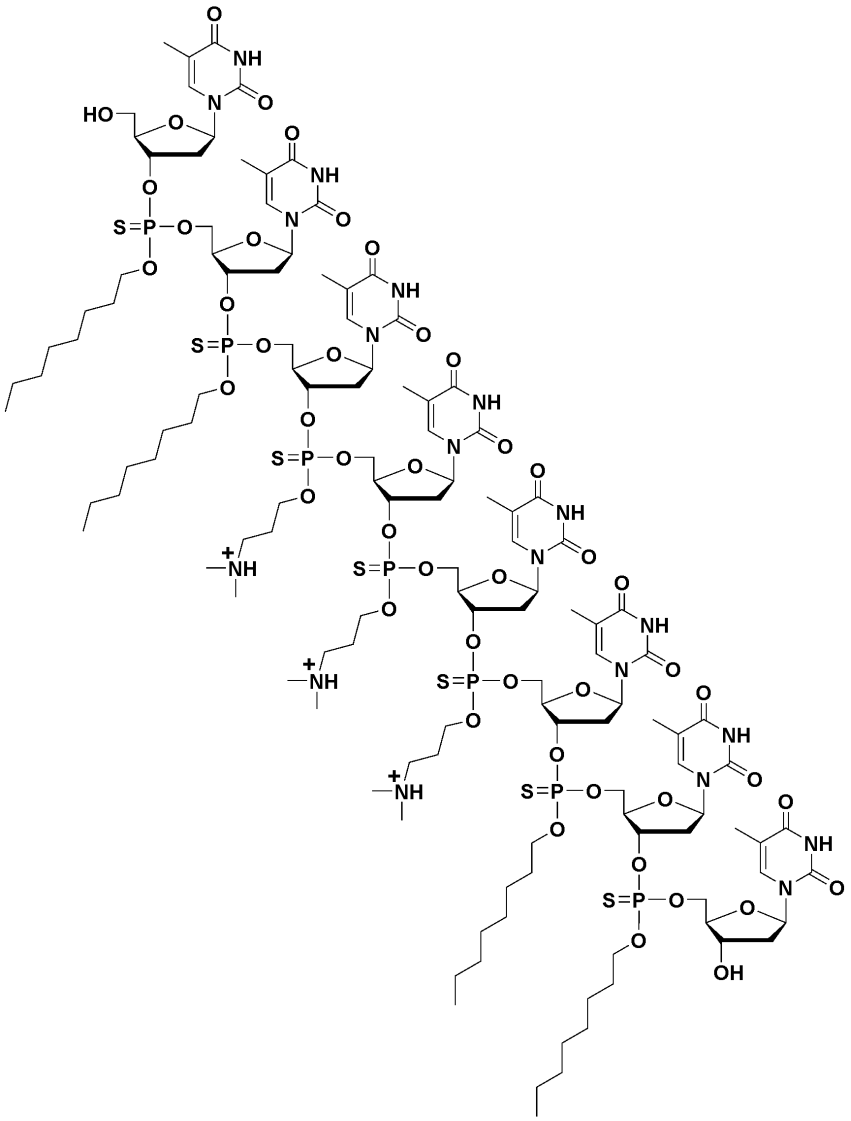
Wu, B., Lu, P., Cloer, C., Shaban, M., Grewal, S., Milazi, S., Shah, S.N., Moulton, H.M., Lu, Q.L. *Am. J. Pathol.* 2012, 181, 392.

A DNA (dTtaPS)- or RNA (2'-OMeUtaPS)-Based Delivery Reagent for PNA and PMO sequences in Mammalian Cells

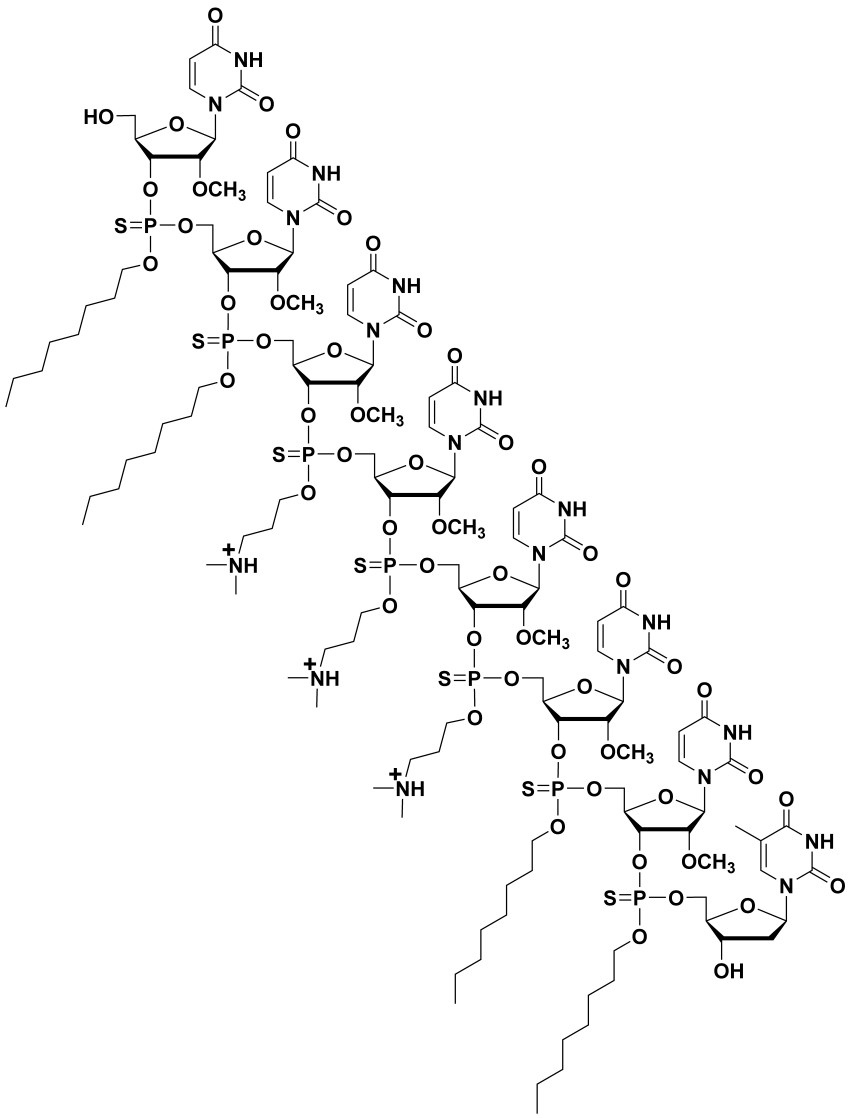
dTtaPS: ***trans*****-acting polythymidylic thiophosphate triester**

2'-OMeUtaPS: ***trans*****-acting poly-2'-*****O*****-methyuridylic thiophosphate triester**

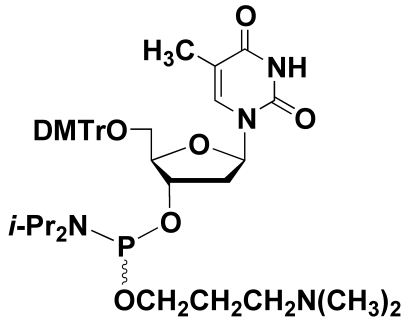
dTtaPS

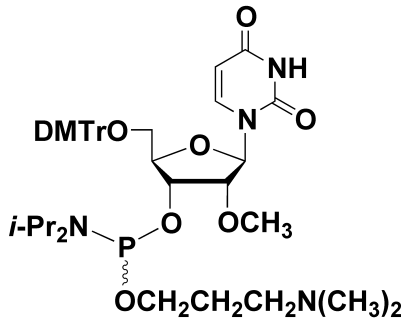


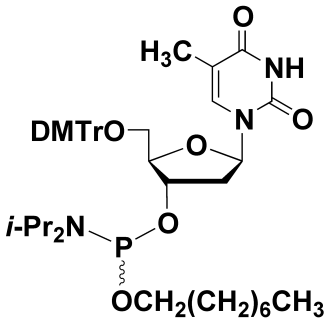
2'-OMeUtaPS

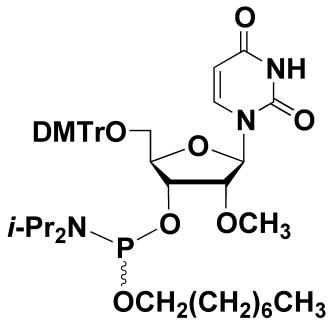


Solid-phase synthesis of dTtaPS and 2'-OMeUtaPS









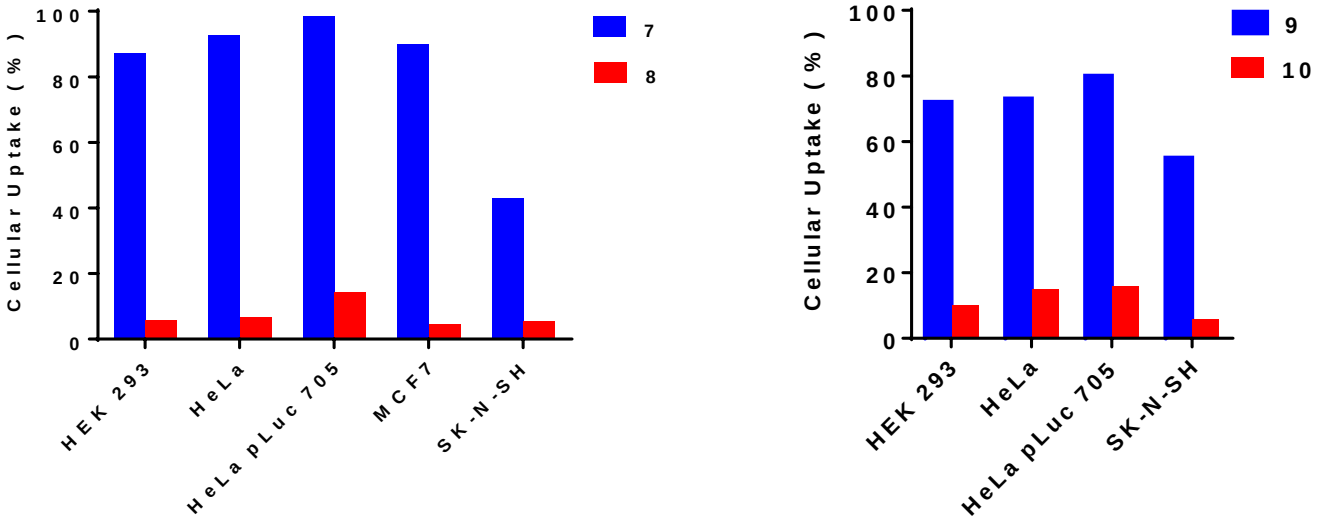
Jain, H.V.; Verthelyi, D.; Beaucage, S.L. *RSC Adv., 2015, 5, 65245*
Jain, H.V.; Boehler, J.F.; Verthelyi, D.; Nagaraju K;. Beaucage S.L. *RSC Adv., 2017, 7, 42519*

Working Hypotheses

Extending PNA or PMO sequences with a short PNA-polyA or PMO-polyA tail could provide an affinity recognition site for a positively charged, dTtaPS or a 2'-OMeUtaPS through sequence complementarity.

In principle, the affinity recognition of polyA-tailed PNA or PMO sequences by dTtaPS or 2'-OMeUtaPS could facilitate the uptake of these sequences in live cells by transiently providing positive charges while avoiding the shortcomings associated with the cellular delivery of cationic CPP-conjugates.

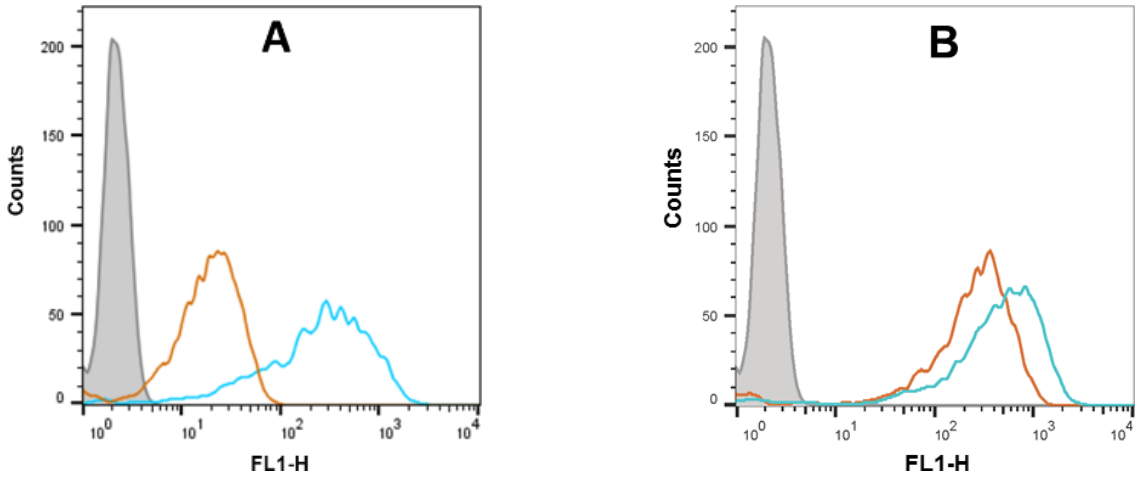
Flow cytometry analysis of the dTtaPS-mediated delivery of PNA or PMO sequences in live cells



PNA sequence 7: Fl-CCTCTTACCTCAGTTACA-AAAAAA-NH_2
PNA sequence 8: Fl-CCTCTTACCTCAGTTACA-NH_2
PMO sequence 9: CCTCTTACCTCAGTTACA-AAAAAA-Fl
PMO sequence 10: CCTCTTACCTCAGTTACA-Fl
Fl:fluorescein

Internalization of the dTtaPS:PNA or dTtaPS:PMO sequence complexes in live mammalian cells. The extracellular concentration of dTtaPS and each PNA sequence (7 or 8) or PMO sequence (9 or 10) is 2.0 μM and 1.0 μM, respectively.

Flow cytometry analysis of the dTtaPS- or 2'-OMeUtaPS-mediated delivery of a PNA or PMO sequence in HeLa pLuc 705 cells

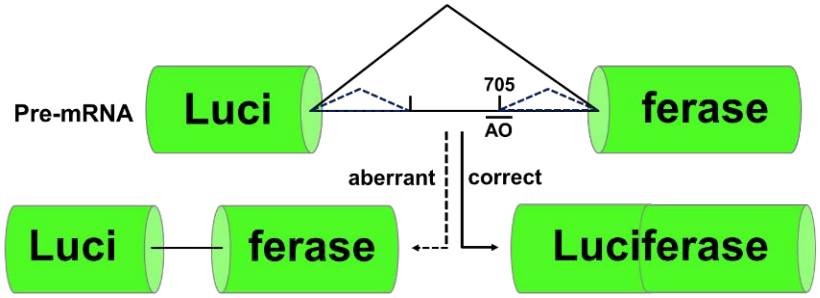


PMO sequence 11: GTGGCCGTTTACGTCGCC-AAAAAA-Fl
PNA sequence 11: Fl-GTGGCCGTTTACGTCGCC-AAAAAA-NH_2
Fl:fluorescein

(A) dTtaPS-assisted cellular uptake of the control PMO sequence 11 (peak area with an orange border) and PNA sequence 11 (peak area with a cyan border)
(B) Same as in (A) but using 2'-OMeUtaPS as the delivery reagent. Gray peak area, untreated HeLa pLuc 705 cells.

The extracellular concentration of dTtaPS or 2'-OMeUtaPS and PMO sequence (11) or PNA sequence (11) is 2.0 μM and 1.0 μM, respectively.

Assessment of the dTtaPS- or 2'-OMeUtaPS-mediated delivery of PNA and PMO sequences in HeLa pLuc 705 cells using a luciferase assay

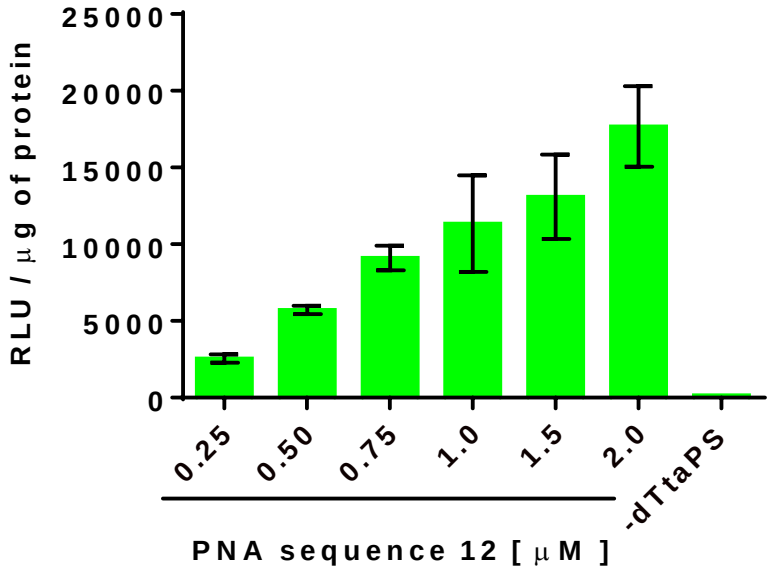


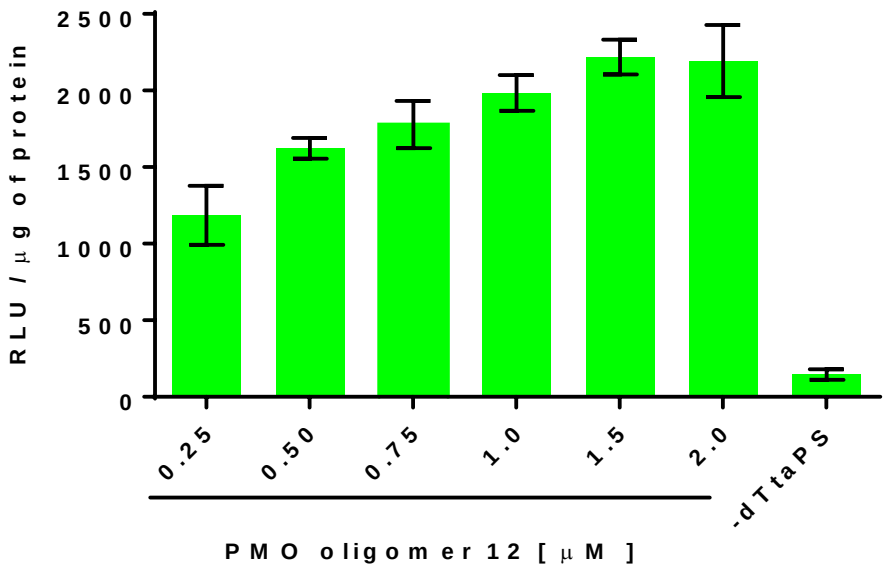
AO: phosphorothioate 2'-*O*-Me RNA sequence complementary to the mutated 705 splice site

- **Plasmid pLuc 705 carries a luciferase cDNA sequence into which a β-globin intron (IVS2-705) has been inserted to interfere with the production of luciferase activity.**
- **In the absence of AO, only basal level of functional luciferase is produced due to incorrect splicing of the modified luciferase pre-mRNA.**
- **In the presence of AO, splicing redirection occurs and restore luciferase function.**
- **Replacing AO with polyA-tailed PNA or PMO results in functional luciferase when dTtaPS or 2'O-MeUtaPS is used as the delivery reagent.**
- **This assay allows one to measure the relative efficiency of uncharged DNA analogues to restore luciferase activity.**

Kang, S. -H; Cho, M. -J.; Kole, R. *Biochemistry* 1998, 37, 6235

Concentration-dependence of the dTtaPS-mediated delivery of a PNA or PMO sequence in HeLa pLuc 705 cells based on luciferase activity production





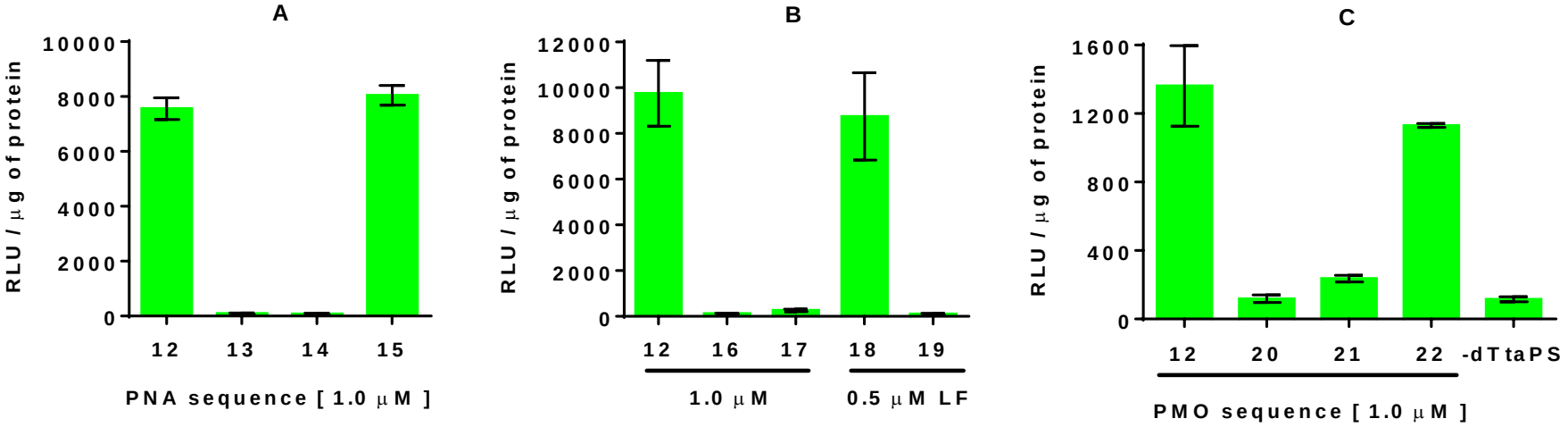
PNA sequence 12: H-CCTCTTACCTCAGTTACA-AAAAAA-NH_2

PMO sequence 12: CCTCTTACCTCAGTTACA-AAAAAA

RLU: relative light units

The concentration of dTtaPS is kept at 2 µM in serum-containing media

A short PNA-polyA or PMO-polyA stretch is necessary and sufficient for the dTtaPS-mediated internalization and Bioactivity of PNA or PMO sequences in HeLa pLuc 705 cells



PNA sequence 12: H-CCTCTTACCTCAGTTACA-AAAAAA-NH_2

PNA sequence 13: H-CCTCTTACCTCAGTTACA-TTTTTT-NH_2

PNA sequence 14: H-CCTCTTACCTCAGTTACA-CCCCCC-NH_2

PNA sequence 15: H-AAAAAA-CCTCTTACCTCAGTTACA-NH_2

PNA sequence 16: H-GTGGCCGTTTACGTCGCC-AAAAAA-NH_2

PNA sequence 17: H-CCTCTTACCTCAGTTACA-NH_2

2'-OMe RNA sequence 18: CCTCTTACCTCAGTTACA (pos. control)

2'-OMe RNA sequence 19: GTGGCCGTTTACGTCGCC (neg. control)

PMO sequence 12: CCTCTTACCTCAGTTACA-AAAAAA

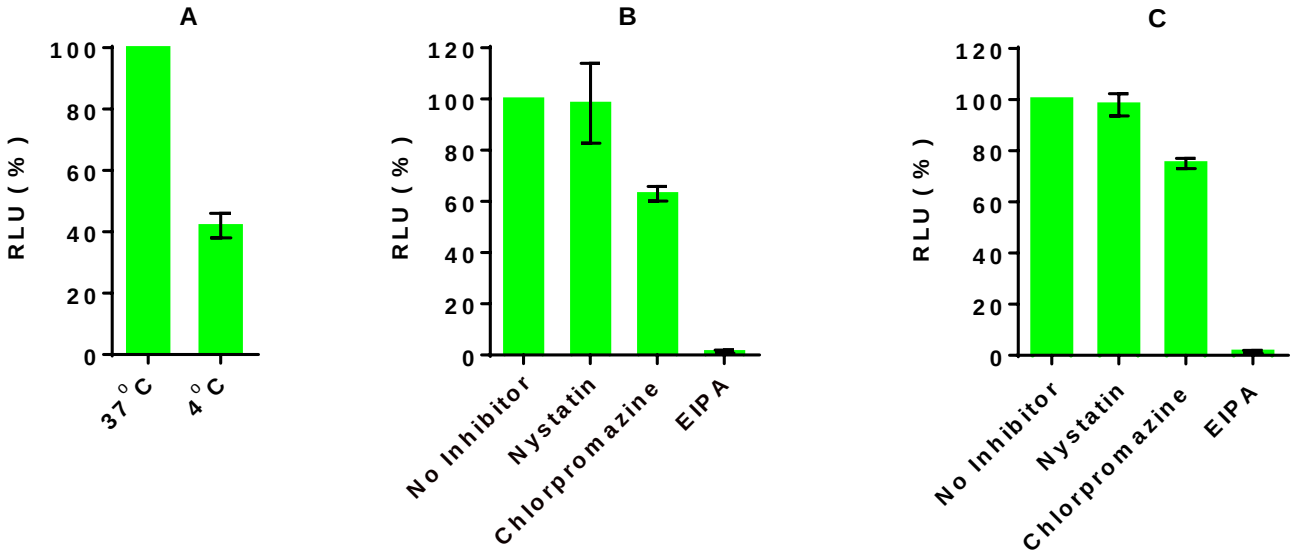
PMO sequence 20: GTGGCCGTTTACGTCGCC-AAAAAA

PMO sequence 21: CCTCTTACCTCAGTTACA

PMO sequence 22: CCTCTTACCTCAGTTACA-AATAAA (mismatch)

LF: Lipofectamine® 2000

Mechanism of the dTtaPS-assisted internalization of a PNA or PMO sequence in HeLa pLuc 705 cells



PNA sequence 12: H-CCTCTTACCTCAGTTACA-AAAAAA-NH_2
PMO sequence 12: CCTCTTACCTCAGTTACA-AAAAAA

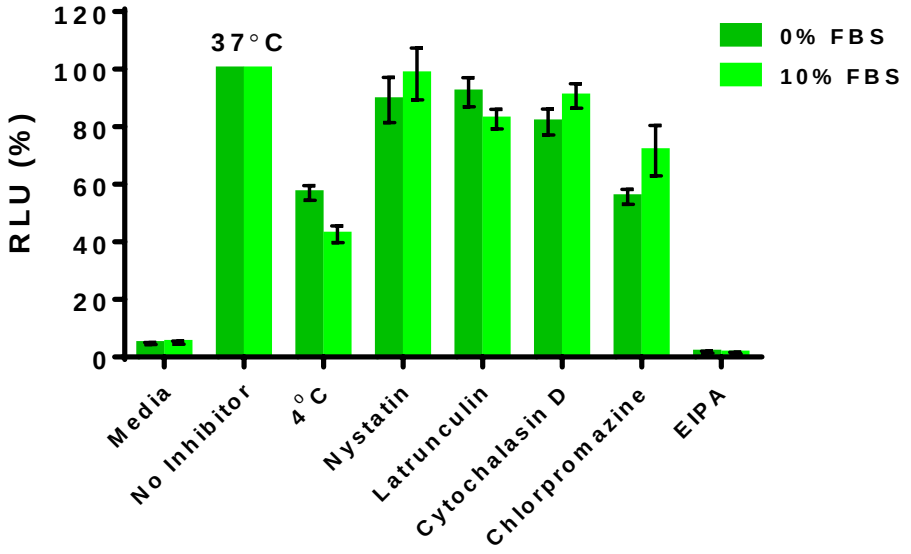
(A) **Temperature-dependence of luciferase activity upon dTtaPS-mediated uptake of PNA sequence 12 in serum-containing medium is indicative of an endocytosis uptake mechanism.**

(B) **Effect of endocytic pathway inhibitors on luciferase activity upon dTtaPS-mediated internalization of PNA sequence 12. Macropinocytosis appears to be the prevailing endocytic pathway for this internalization process.**

(C) **Effect of endocytic pathway inhibitors on luciferase activity upon dTtaPS-mediated internalization of PMO sequence 12. Macropinocytosis also appears to be the prevailing endocytic pathway for this internalization process.**

Nystatin inhibits caveolae-mediated endocytosis, Chlorpromazine inhibits clathrin-coated pits-mediated endocytosis, and 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA) inhibits macropinocytosis.

Mechanism of the 2'-OMeUtaPS-assisted internalization of a PMO sequence in HeLa pLuc 705 cells

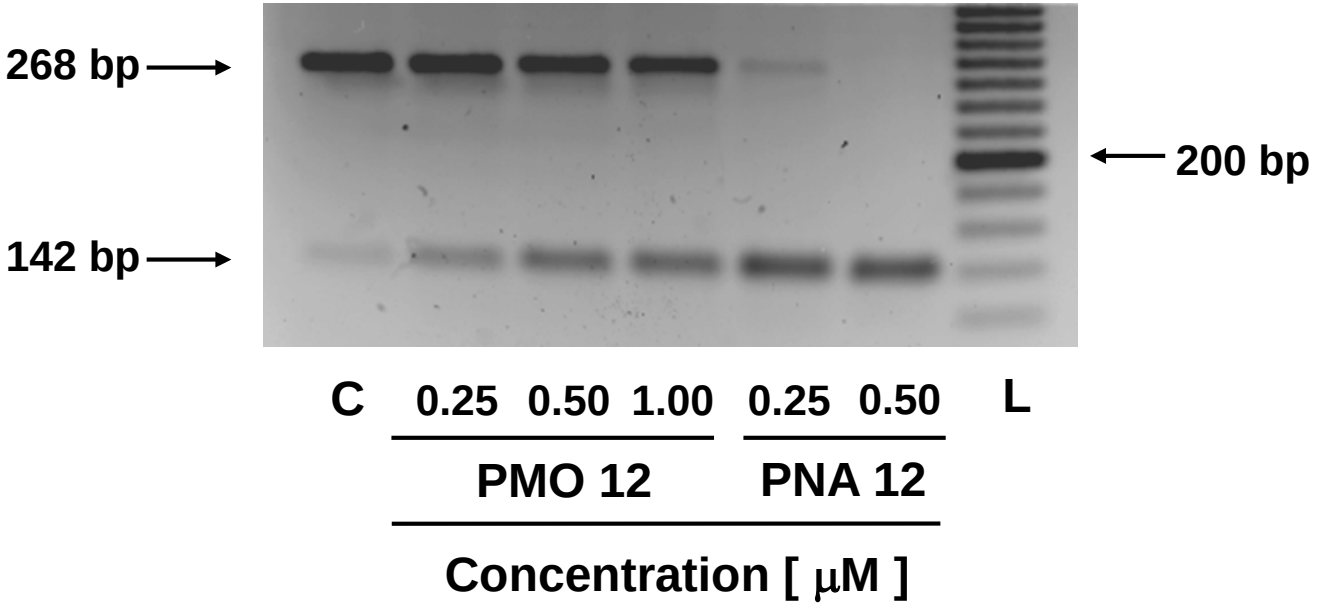


PMO sequence 12: CCTCTTACCTCAGTTACA-AAAAAA

Temperature-dependence and effect of endocytic pathway inhibitors on luciferase activity upon 2'-OMeUtaPS-mediated internalization of PMO sequence 12 in serum-free or serum-containing medium is indicative of an endocytosis uptake mechanism; macropinocytosis appears to be the prevailing endocytic pathway for this internalization process.

Nystatin inhibits caveolae-mediated endocytosis, Chlorpromazine inhibits clathrin-coated pits-mediated endocytosis, and 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA) inhibits macropinocytosis.

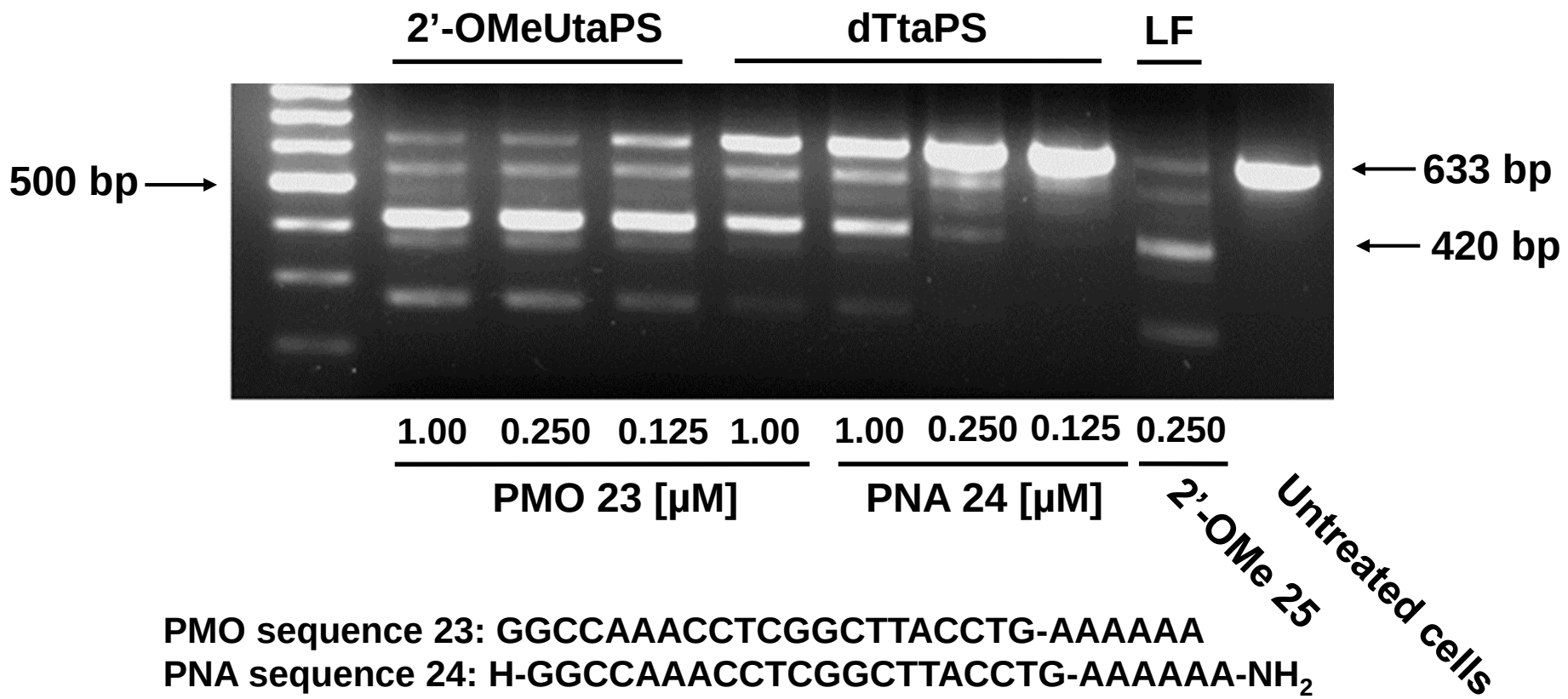
Dose-dependence of splice correction events induced by the dTtaPS-mediated delivery of a PMO or PNA sequence into HeLa pLuc 705 cells



PNA sequence 12: H-CCTCTTACCTCAGTTACA-AAAAAA-NH_2
PMO sequence 12: CCTCTTACCTCAGTTACA-AAAAAA

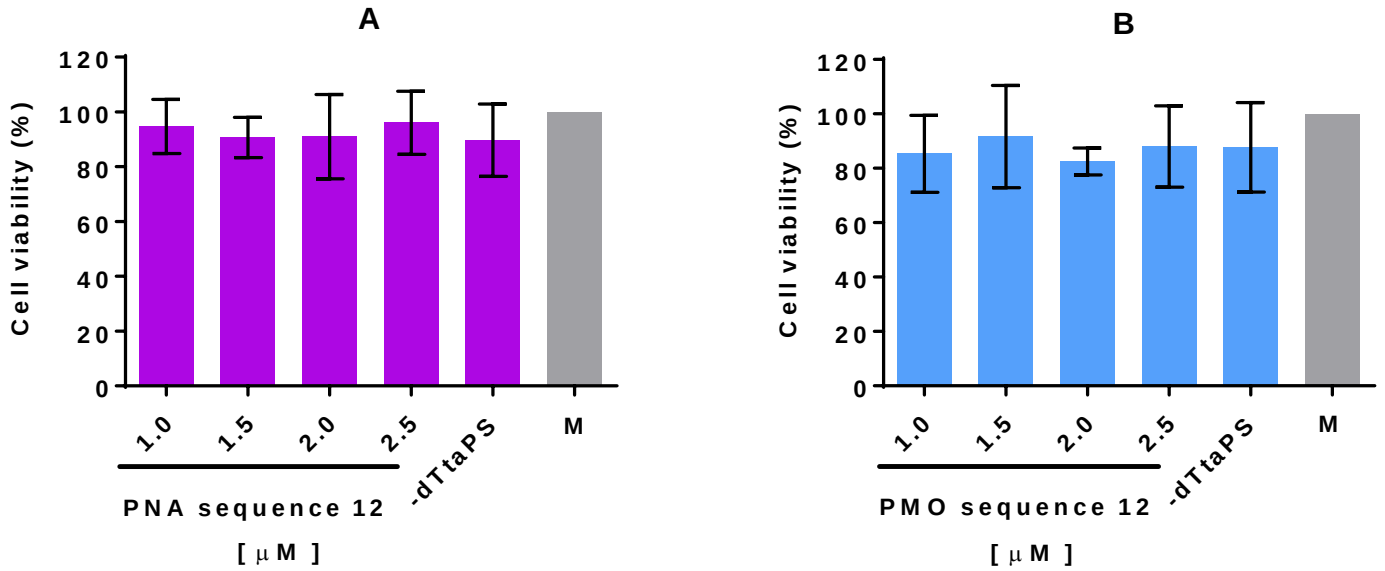
Agarose gel analysis of amplified RT-PCR products obtained from incorrectly (268 bp) and correctly (142 bp) spliced luciferase pre-mRNA

Efficiency of 2'-OMeUtaPS and dTtaPS at inducing the excision of exon 23 from the *mdx* mouse dystrophin pre-mRNA upon transfection of a PMO or PNA sequence in *mdx* mouse myotubes



PMO sequence 23: GGCCAAACCTCGGCTTACCTG-AAAAAA
PNA sequence 24: H-GGCCAAACCTCGGCTTACCTG-AAAAAA-NH_2
2'OMe-RNA sequence 25: GGCCAAACCUCGGCUUACCUGAAAU

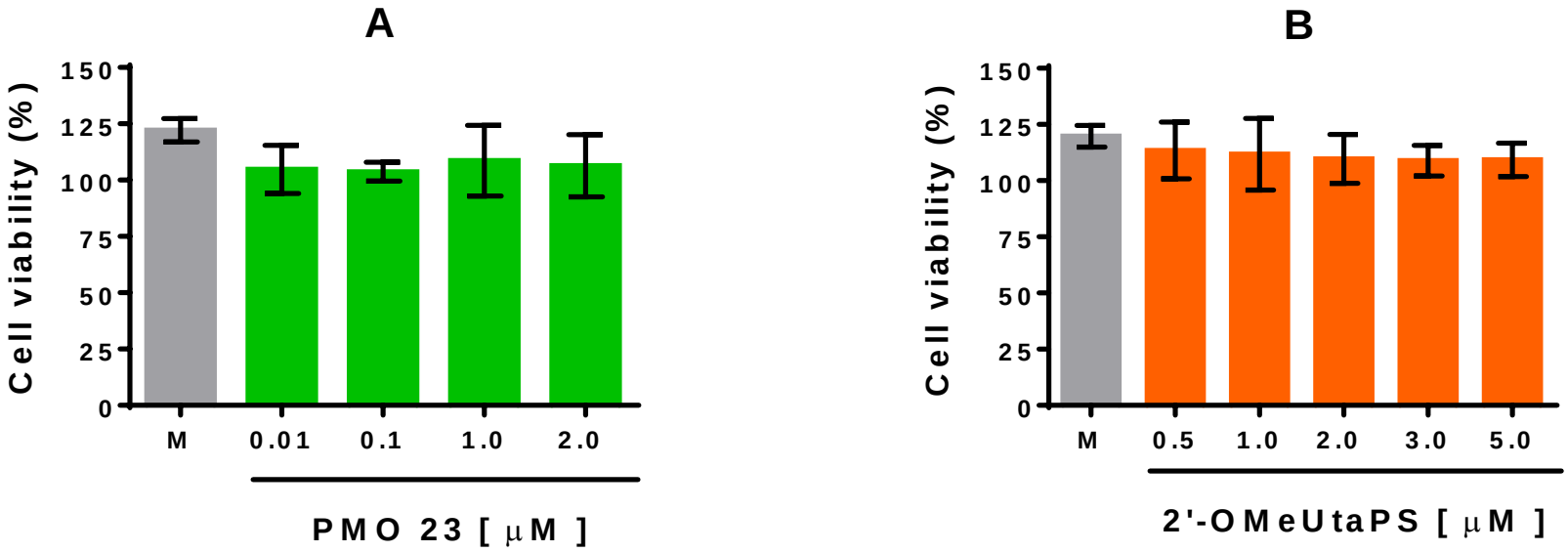
Cytotoxicity of PNA sequence:dTtaPS or PMO sequence:dTtaPS complexes in HeLa pLuc 705 cells



PNA sequence 12: H-CCTCTTACCTCAGTTACA-AAAAAA-NH_2
PMO sequence 12: CCTCTTACCTCAGTTACA-AAAAAA

Increasing the concentration of the PNA or PMO sequence 12 from 1.0 μM to 2.5 μM while keeping the concentration of dTtaPS at 2.0 μM did not induce significant cytotoxicity when compared to that of the medium (M) or in the absence of dTtaPS.

Cytotoxicity of the PMO sequence and 2'-OMeUtaPS in HeLa pLuc 705 cells



PMO sequence 23: GGCCAAACCTCGGCTTACCTG-AAAAAA

Each cytotoxicity study was evaluated over a period of 18 h in serum containing (10% FBS) DMEM medium.

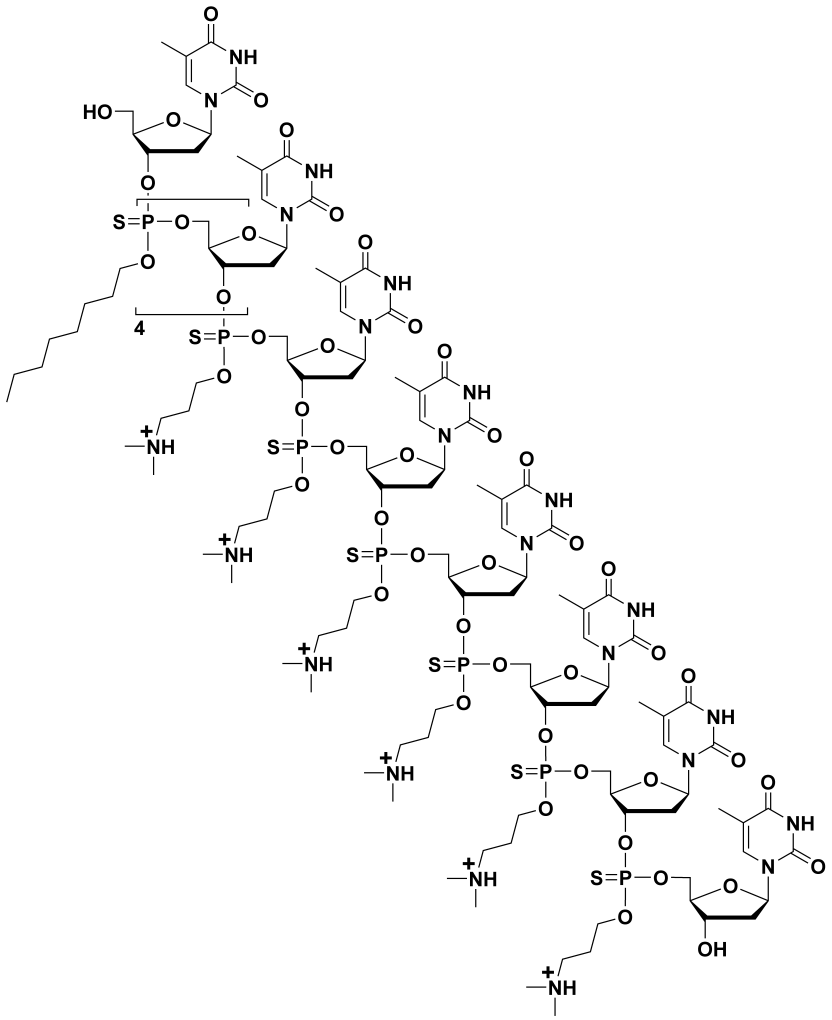
Error bars represent the mean ± SD of three independent experiments. M, medium.

Collaborators

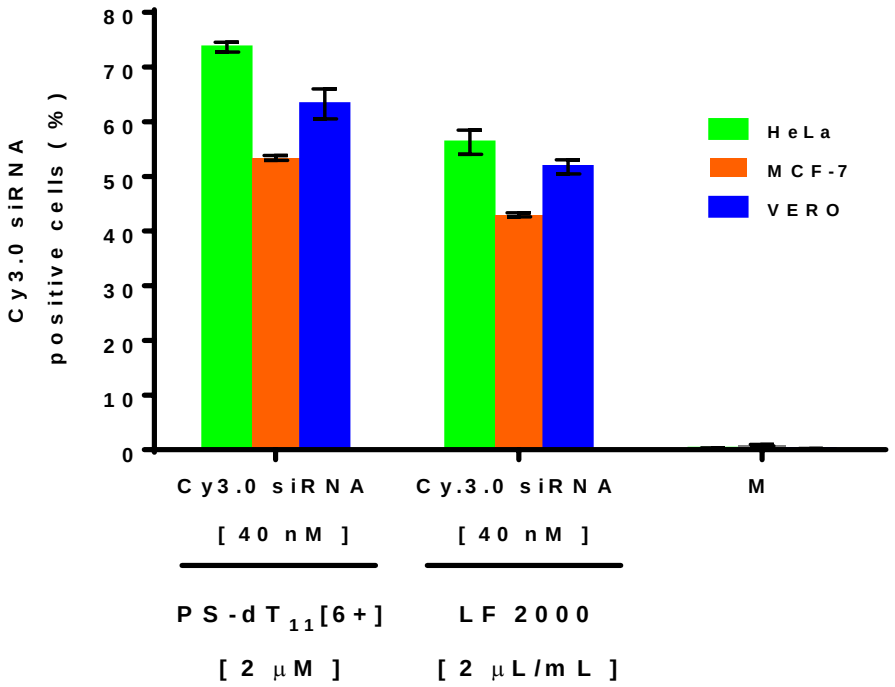
Harsh Jain

Daniela Verthelyi

An amphipathic *trans*-Acting phosphorothioate DNA sequence delivers a siRNA duplex in mammalian cells

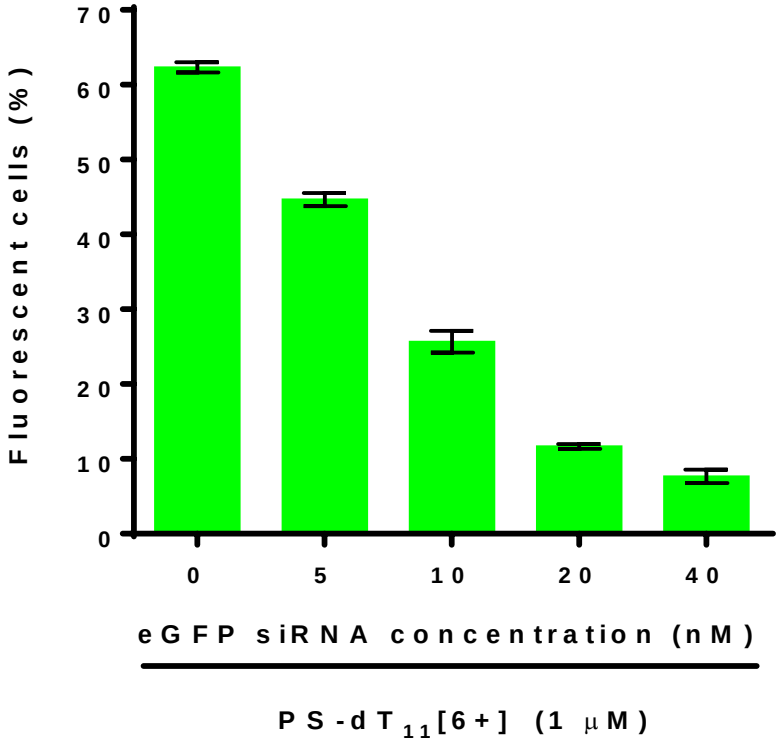


PS-dT$_{11}$[6+]



FACS analysis of the PSpdT$_{11}$[6+]-mediated delivery of the Silencer Cy3.0-labeled GAPDH siRNA in mammalian cells

Concentration-dependence of eGFP knockdown upon PS-dT$_{11}$[6+]-mediated delivery ofSilencer™GFP siRNA in Vero cells



% of fluorescent cell measurements were obtained from three independent experiments performed in serum-containing medium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