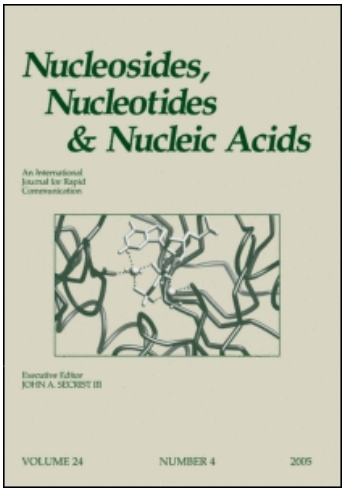

Nucleosides, Nucleotides and Nucleic Acids

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Confocal-microscopy Studies of a Model Oligoribonucleotide HIV Inhibitor

Robyn M. Hyde[a]; Keith Jensen[b]; Jindrich Kopecek[b]; Arthur D. Broom[c]
[a] Westminster College, Salt Lake City, Utah, USA [b] Department of Pharmaceutics & Pharmaceutical Chemistry, University of Utah, Salt Lake City, Utah, USA [c] Department of Medicinal Chemistry, University of Utah, Salt Lake City, Utah, USA

To cite this Article Hyde, Robyn M. , Jensen, Keith , Kopecek, Jindrich and Broom, Arthur D.(2005) 'Confocal-microscopy Studies of a Model Oligoribonucleotide HIV Inhibitor', Nucleosides, Nucleotides and Nucleic Acids, 24: 10, 1875 — 1884
To link to this Article: DOI: 10.1080/15257770500268913
URL: http://dx.doi.org/10.1080/15257770500268913

Nucleosides, Nucleotides, and Nucleic Acids, 24:1875–1884, 2005

ISSN: 1525-7770 print/1532-2335 online
DOI: 10.1080/15257770500268913

Taylor & Francis
Taylor & Francis Group

CONFOCAL MICROSCOPY STUDIES OF A MODEL OLIGORIBONUCLEOTIDE HIV INHIBITOR

Robyn M. Hyde □ *Westminster College, Salt Lake City, Utah, USA*

Keith Jensen and Jindrich Kopecek □ *Department of Pharmaceutics & Pharmaceutical Chemistry, University of Utah, Salt Lake City, Utah, USA*

Arthur D. Broom □ *Department of Medicinal Chemistry, University of Utah, Salt Lake City, Utah, USA*

□ *Previous work has shown that novel amphipathic oligo and polyribonucleotides are potent inhibitors of HIV. It was hypothesized that the mechanism(s) of action for these compounds might be inhibition of retroviral reverse transcriptase (RT) and/or viral uptake by cells. A fluorescent oligonucleotide analog was prepared, and confocal microscopy studies were undertaken in order to examine oligonucleotide-cell interactions.*

Keywords Oligonucleotide; Amphipathic; HIV inhibitor; Confocal microscopy; Cell uptake

INTRODUCTION

The search for new drugs to combat the Human Immunodeficiency Virus (HIV) is becoming increasingly important due to the development of resistance towards current therapeutics.[1] At present, there are four classes of drugs available; namely, the nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), and fusion inhibitors (FIs) (Table 1). These are used in various combinations in an attempt to block viral proliferation and to minimize the serious toxicities that often arise.[2] This therapeutic strategy is known

In honor and celebration of the life and career of John A. Montgomery.

Received 17 February 2005; accepted 23 May 2005.

These studies were supported by research grants R01 AI27692 from the NIAID, NIH, PHS (ADB) and CA51578 (JK). The authors are grateful for the synthesis of OAI-fl in the DNA/Peptide Synthesis Core Facility of the Huntsman Cancer Institute supported by CA42041.

Address correspondence to Arthur D. Broom, Department of Medicinal Chemistry, University of Utah, 30 S. 2000 E, Room 201 Skaggs Hall, Salt Lake City, UT 84112. Fax: (801) 581-3716; E-mail: abroom@deans.pharm.utah.edu

TABLE 1 Current HIV Therapeutics

Protease inhibitors (PI)	Nucleoside RT inhibitors (NRTIs)	Nonnucleoside RT inhibitors (NNRTIs)
Amprenavir (Agenerase)	Abacavir (Ziagen)	Delavirdine (Rescriptor)
Atazanavir (Reyataz)	Didanosine (Videx, ddI)	Efavirenz (Sustiva)
Fosamprenavir (Lexiva, 908)	Emtricitabine (Emtriva FTC)	Nevirapine (Viramune)
Indinavir (Crixivan)	Lamivudine (Epivir, 3TC)	
Lopinavir/Rotonavir (Kaletra)	Stavudine (Zerit, d4T)	
Nelfinavir (Viracept)	Tenofovir DF (Viread)	Fusion inhibitors
Ritonavir (Norvir)	Zalcitabine (Hivid, ddC)	Enfuvirtide (Fuzeon, T-20)
Saquinavir (Fortovase, Invirase)	Zidovudine (Retrovir, AZT, ZDV)	

as highly active anti-retroviral therapy or HAART. Even with this combination therapy, however, the clinical manifestation of the Acquired Immune Deficiency Syndrome (AIDS) is inevitable for patients because eventually the drug pressure selects for resistant strains that then proliferate. The resistance problem is further compounded by the fact that there is significant cross-resistance between drugs in each class, often making second-line therapy ineffective.[1]

Investigations leading toward new therapeutic options have generally taken one of two directions. While some investigations have emphasized development of analogs of the current anti-retroviral medicines such as the NRTIs and PIs, others have focused on identifying inhibitors of new targets within the virus life-cycle.[3]

Work in this lab has focused on the development of novel oligo- and polyribonucleotides as a new class of anti-retroviral drugs (Figure 1). Homopolymers of unusually modified nucleic acids have been shown to inhibit RT in cell-free extracts, to act at a very early stage in the viral life cycle, and to protect cells against the pathogenicity of HIV.[4–8] These nucleic acid macromolecules are considered to be "senseless" in nature in that they have no Watson-Crick hydrogen bonding potential. They are also amphipathic, with a hydrophilic backbone and hydrophobic base. These and related oligo- and polynucleotides have been suggested by Bardos and his colleagues to act as "anti-templates" by binding in the reverse transcriptase (RT) template-binding site and blocking normal reverse transcription.[9] Kinetic studies based on time-of-addition of drug to cell cultures seem consistent with inhibition of very early events in the virus life cycle, perhaps binding, fusion or uncoating, but do not eliminate the possibility of dual action as RT inhibitors.[10]

One such senseless oligonucleotide is a 33-mer produced by machine synthesis consisting of a single 5′-unprotected thymidine attached to the solid support, to which was added 32 residues of 1-allyl-2′-O-methylinosinic acid (Figure 1).[8] This molecule, designated OAI for oligo-(1-allyl-2′-O-

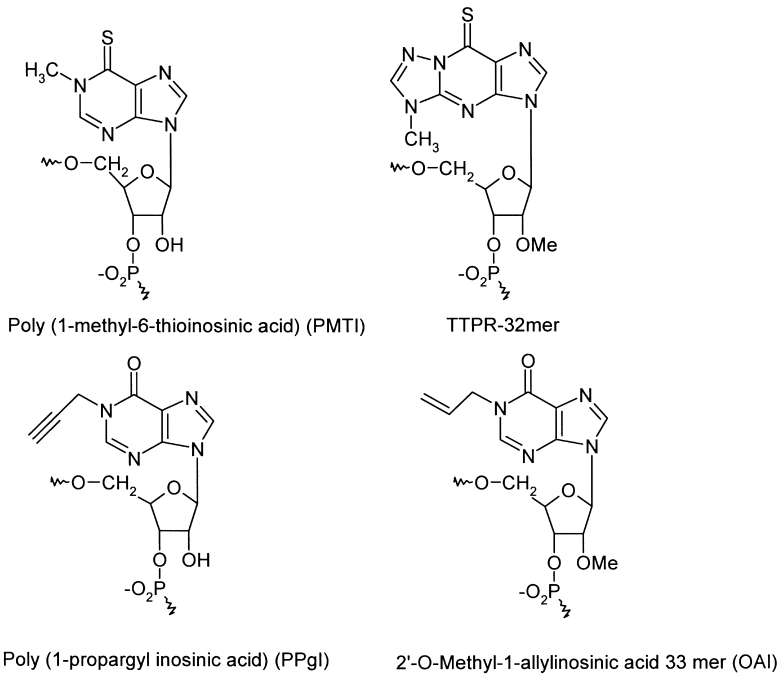

FIGURE 1 Unusually modified homopolymer constructs.

methylinosinic acid), was found to inhibit the HIV cytopathic effect at submicromolar concentrations.[8] For the present study, a similar construct was prepared, differing only in the substitution of a fluorescein derivative at oligonucleotide positions 7 and 28 (Figure 2) and designated OAIfl.

Kinetic and whole cell studies have shown that the prototype compound that led to these studies, poly (1-methyl-6-thioinosinic acid) (PMTI), behaves as if it has multiple mechanisms of action that could include viral uptake inhibition as well as RT inhibition.[9] To approach the important question of cell uptake vs. cell surface binding, studies using confocal microscopy were undertaken using the PMTI analog OAIfl.

Confocal microscopy is a powerful technique used by many investigators in a variety of fields to help answer mechanism of action questions. This technique has been used to elucidate the mechanisms of drug toxicity,[10] drug resistance,[11] gene delivery,[12] and to investigate vaccination effectiveness[13] and the potential of new drugs.[14] Additionally, it has been used in other HIV studies to reveal the roles of the accessory proteins,[15] outline the HIV entry process,[16,17] and describe the process of budding.[18,19] It generally involves the visualization of a fluorescently labeled "probe" within a biological system.

It was thought that this technique would provide a better defined picture of the oligonucleotide-cell interactions vis-à-vis surface binding, cell entry,

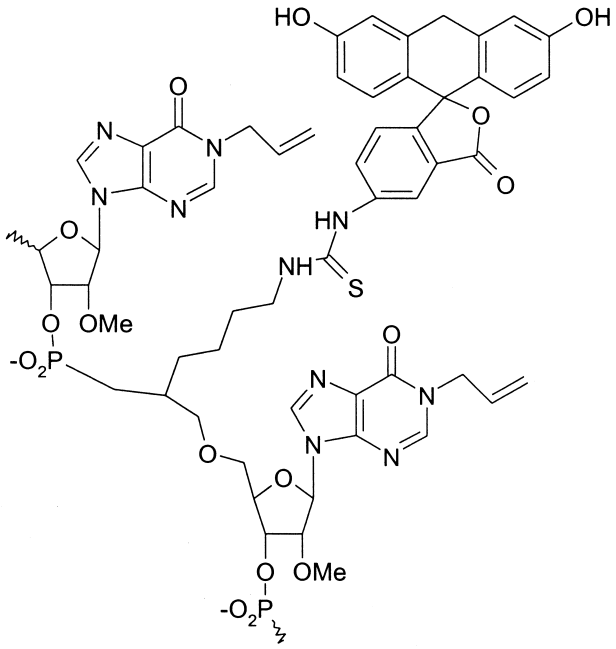

FIGURE 2 Fluorescently labeled model oligonucleotide probe (OAIfl).

and cytoplasmic vs. nuclear localization. The time course of these events could then be correlated with previous time-of-addition studies to assist in the evaluation of the mechanism of action hypotheses presented above. If the hypotheses of drug activity are as proposed (both cell uptake and RT inhibition), the oligonucleotide probe should demonstrate cell surface binding and fairly rapid cytoplasmic internalization. The study had two main objectives: (1) to determine the time course of drug uptake and (2) to determine the method of drug entry (diffusion vs. endocytosis).

RESULTS AND DISCUSSION

The confocal microscopy work was conducted in a cell line derived from the CEM T4-lymphoblastoid cell line normally used in HIV inhibition studies. It was important for this study to model drug uptake in the type of cells normally infected by HIV. The particular cell line used was the 1A2[20] cell line obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program. The 1A2 cells are constitutive Tat and Rev-producing lymphocyte cells. This allows HIV work to be conducted in a level 2 biological facility using the Tat and Rev deficient virion HIV MC99D Tat-Rev. Extensive work has shown that these experimental conditions give inhibition values for FDA approved and experimental drugs comparable to the T4 lymphocytic cell line CEM-SS.[21]

Because 1A2 cells are suspension cells, they do not spontaneously adhere to the microscope slide as required for confocal microscopy. Centrifugation was required to obtain an adherent population of cells. In brief, the cells, after treatment with the labeled oligomer for a predetermined time at 37°C, were fixed with paraformaldehyde, transferred from a 96 well plate into a Lab Tek II chamber slide system (Nalge-Nuc), centrifuged and sealed to the slide with a cover slip.

Though the slide system was not designed to be used with centrifugal force, the product withstood this procedure quite well. Since centrifugal force can lead to cell lysis, the rotor speed was closely monitored. A methylthiazoletetrazolium (MTT) assay to asses the cell viability following drug treatment and centrifugation along with microscopic examination of overall cell morphology showed that the cells remained viable.

The 1A2 cells were treated with 50, 250, or 1000 nM solutions of fluorescently tagged 2′-O-methyl-1-allylinosinic acid 33-mer (OAIfl, Figure 2) in PBS for 0.25, 1, 2, 4, or 8 h. Since the purpose of the confocal microscopy study was to help identify potential inhibition mechanisms used by this class of drugs, the concentrations of OAIfl used approximated the therapeutic dosages observed in earlier studies with other homopolymers and oligomers.[7,8] It seemed likely that if very high doses were used, though the pictures taken might be exceptional in quality, the cellular uptake and distribution patterns might be altered and less informative.

The fluorescent data clearly show that at 0.25 h, there was fluorescent material associated with the cell membrane (Figure 3).[22] The fluorescence at this earliest time point was weak and limited to the periphery of the cell, but was clearly discernable. This rapid cell surface interaction supports the hypothesis that viral entry inhibition may play a significant role in the anti-HIV activity of these compounds.

Over the full time course of the study, the fluorescence intensity increased both peripherally and internally. Due to the polyanionic nature of the backbone, it was predicted that the compounds would not passively diffuse through the cellular membrane but would be internalized by endosomal activity. At early time points, beyond 1 h and before 4 h, the fluorescence pattern was primarily punctate. This indicates that the primary route of internalization is, indeed, by endosomal vesicle inclusion. At 2 h, though the fluorescence observed is still primarily punctate, it appears that some of the fluorescent drug has escaped from the vesicles and has entered the cytoplasm. At 4 h, the intensity of the fluorescence in the cytoplasm has significantly increased. Though the amount of drug internalized is very difficult to quantitate, it appears that prior to 4 h there could be sufficient concentration within the cytoplasm to effect RT inhibition. The photomicrographs taken at 8 h continue to show increasing amounts of fluorescence both within the endosomal vesicles and the cytoplasm. Additionally, very bright spots beyond the emission scale became apparent.

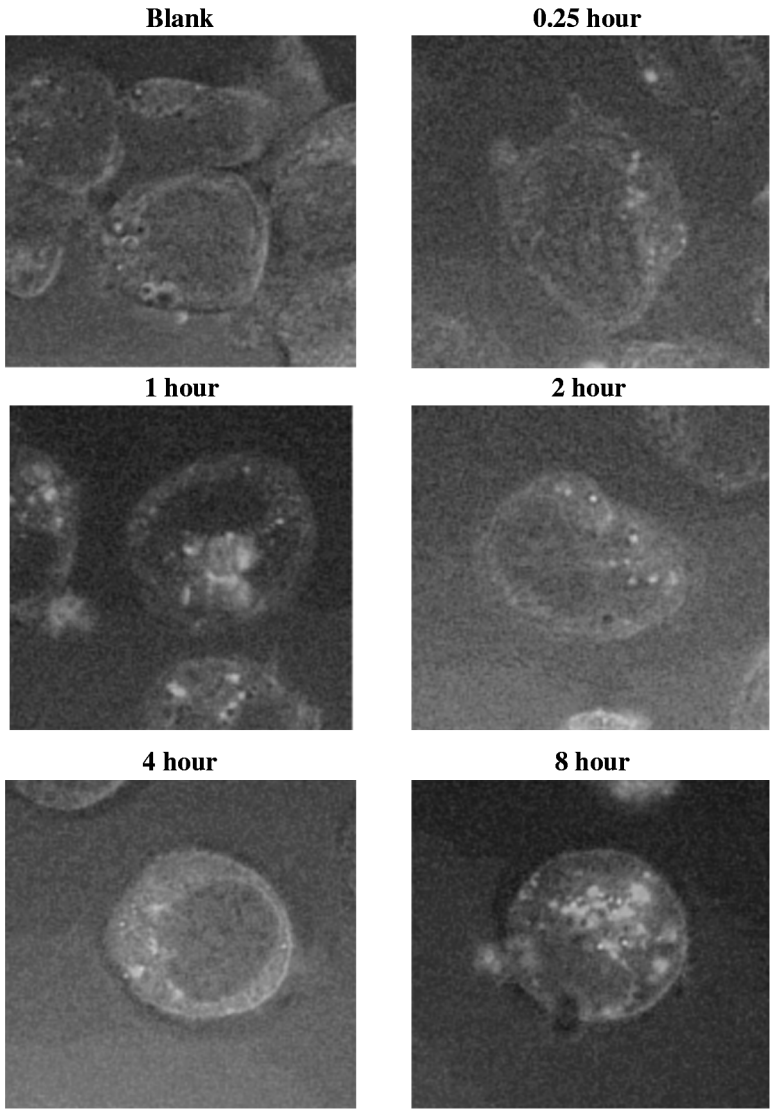

FIGURE 3 Time course confocal microscopy of fluorescent 2′-O-methyl-1-allylinosine 31-mer (OAIfl) in 1A2 cells at 250 nM.

It is important to note that at no time, even at the highest treatment concentration, was fluorescence observed within the nucleus. Clearly, the drug was unable to penetrate the nuclear membrane. This helps to substantiate the hypothesis that inhibition of HIV by these compounds must be in the early stages of the life cycle. Following the early steps viral entry, uncoating and reverse transcription, the viral life cycle is contained within the nucleus until the later steps of viral translation and packaging, which are accom-

plished in the cytoplasm. Previous time of addition studies performed with poly(1-methyl-6-thioinosinic acid) (PMTI, Figure 1) seemed to preclude the late life cycle steps as being targeted by drugs in this class.[9] It was found that treatment with PMTI 4 h or more after infection had no therapeutic effect. After 4 h, it is assumed that all early steps in the life cycle up through viral reverse transcription have been completed.

It is of some interest that the time of addition studies indicate that PMTI has little activity 4 h after viral infection and that the confocal microscopy study with OAIfl indicates that cytoplasmic concentration of drug gradually increases during this time period. Though the confocal microscopy data are fully consistent with the potential for uptake inhibition, the cellular accumulation times neither clearly support nor negate action as an RT inhibitor. Based on the reasonable assumption that the drug uptake profile and mechanism of actions were similar for OAIfl and PMTI, the time-of-addition studies do not conclusively indicate that cytoplasmic drug concentrations will achieve RT inhibitory levels prior to 4 h after administration. While the primary mode of action of OAI and, by implication, other compounds in this series, may be as entry inhibitors, inhibition of RT is not excluded as a reasonable mechanism for these molecules.

In conjunction with this study, it was thought that it would be important to verify that the fluorescence observed in the cells was due to the internalization of intact drug and not of smaller oligomers resulting from serum degradation. HPLC analysis of oligomers incubated with serum up to 48 h indicated that there was only limited oligonucleotide degradation within this time period. HPLC analysis of the oligomers was conducted with an "in-line internal surface reverse-phase cleaning" (ISRP) column[23] in-line with a C8 column.[24]

EXPERIMENTAL

Fluorescein Labeled 2′-O-Methyl-1-allylinosinic Acid 33-Mer, OAIfl. A 33-mer oligomer was synthesized from 5′-O-(4,4′-dimethoxytrityl)-2′-O-methyl-1-allylinosine phosphoramidite[8] on a 1 μmol scale with an automated DNA synthesizer. Positions 7 and 28 of the oligomer were substituted with a fluorescein phosphoramidite. Crude DMTr-ON product was dried by speed evaporation at room temperature and purified by the Poly-Pac II cartridge purification scheme developed by Glen Research. Following cartridge preparation with acetonitrile (4 mL) and ammonium acetate (2.0 M, 4 mL), the cartridge was flushed three times with the DMTr-ON oligo solution (3 mL, 0.1 M triethyl ammonium acetate). The cartridge was washed with 6 mL NH_4OH (1:10) followed by 4 mL H_2O. Oligonucleotide was detritylated by flushing the column with 2% TFA (4 mL) and following a water wash (4 mL), the DMTr-OFF product was eluted with acetonitrile/water (1:1, 4 mL).

Collected product was lyophilized and converted to Na^+ form by again using the Poly Pac-II cartridge. For Na^+ conversion, the cartridge was prepared with acetonitrile (4 mL) and sodium acetate (2.0 M, 4 mL) and the DMTr-OFF product was prepared by dissolution in 3 mL 0.1 M sodium acetate. DMTr-OFF oligonucleotide was loaded onto the cartridge by flushing three times with the solution. The cartridge was washed with 6 mL of sodium acetate (0.1 M) and the oligonucleotide was eluted as the Na^+ salt with acetonitrile/H_2O (1:1, 4 mL). Collected product was lyophilized, dissolved in 5 mL H_2O and dialyzed against 0.1 M NaCl (24 h, 8 L) and against H_2O (48 h, 16 L). Lyophilized product yielded 7.3 mg (56.4%) of a bright yellow fluffy solid. UV: ε_{max} 8010 at 250 nm; MALDI (M+Na) calcd 12951, found 12945; HPLC retention time 22.2 min (reverse phase protocol); homogeneous by HPLC.

Confocal Microscopy Assay[25]. 1A2 cells acquired from the NIH AIDS Research and Reference Reagent Program were grown in media containing RPMI 1640 media and 80% FBS. Cells were split every 2–3 days when concentration reached 1×10^6 to 5×10^6 cells/mL. Before analysis, cells were diluted with fresh media. Each test and control well in a 96-well plate were filled with 5×10^4 cells. Fluorescently labeled OAI (OAIfl) that had been brought up in sterile DPBS was added to test wells to a 1000 nM, 250 nM, or 50 nM final concentration. After predetermined amounts of time (15 min, 1 h, 2 h, 4 h, and 8 h), the reaction was terminated by washing all drug from the cells. Cells were centrifuged at 1000 rpm at room temperature for 10 min. The medium was aspirated from the cell pellet carefully and the cells were resuspended in 0.2 mL DPBS.

To prepare for confocal microscopy, the cells, once aspirated of DPBS, were fixed in 3% paraformaldehyde (in DPBS) for 20 min, centrifuged, and aspirated. The cells were then resuspended in DPBS, transferred to an 8-well chamber slide, centrifuged and aspirated. In a final step, the cells were treated with the anti-fade reagent SlowFade Light as recommended by the manufacturer and the cells were sealed to the slide with a cover slip using Cytoseal 60.[26]

The cell images were visualized on a Zeiss (Thornwood, NY) LSM 510 confocal imaging system with an Axioplan 2 microscope (100× plan-apo objective, NA = 1.4, oil) using an argon laser (excitation 488 nm, emission 505 nm long-pass filter). Analysis of the images was performed on the Zeiss 510 Image Browser program (version 2.30.011). The gain setting was adjusted so that the blank control cells only showed minimal autofluorescence. The images (8-bit fluorescence) were scaled to 256 gray levels and a green look up table (LUT) was applied.

MTT Assay. 1A2 cells were prepared as previously described and treated with 50 nM, 250 nM, 1,000 nM, and 10,000 nM solutions of OAIfl.

The cells were treated as in the confocal microscopy assay, except, the paraformaldehyde wash was substituted with a DPBS wash and cells were not transferred to a slide chamber. MTT (20 μL) was added to each well and after an overnight incubation were lysed with 5 μl DMSO and gently swirled. MTT absorbance was read with a BioRad 450 microplate reader.

HPLC Degradation Assay. OAI was incubated with media containing Roswell Park Memorial Institute (RPMI) 1640 media 1620 and 20% fetal bovine serum (FBS) at 37°C. At 10 and 48 h, sample aliquots were removed and analyzed by the ISRP method. Initially after sample injection, the large biomolecules from the media were eluted isocratically from the ISRP column (ChromSpher BioMatrix) by Varian with 0.1 M ammonium acetate. At 3 min, a reverse-phase C8 column was added in-line and adsorbed small molecules were eluted by gradient with acetonitrile (60%, 48 min; phase a: 0.1 M ammonium acetate; phase b: acetonitrile).

CONCLUSIONS

It has been well established over a number of years that so-called senseless oligo- and polyribonucleotides are potent anti-HIV agents, but their mechanism of action has not been clearly established. The present study makes significant progress toward understanding how these compounds exert their activity by the use of a fluorescently-labeled oligonucleotide, OAIfl, to evaluate the time course of cell binding and uptake. Surface binding was found to be very rapid, with detectable association within 15 min. Internalization within endosomal vesicles was also fairly rapid; cytoplasmic levels increased steadily for several hours of incubation of cells with drug. These data strongly suggest that the initial mode of action is inhibition of some stage of virus uptake. Because it was not possible to assess cytoplasmic drug concentrations quantitatively (the extent of fluorescence quenching or enhancement within the cytoplasm is unknown), the possibility that RT inhibition may occur through endosomal leakage of drug early in the course of drug uptake cannot be excluded.

REFERENCES

1. Johnson, V.A.; Brun-Vézinet, F.; Clotet, B.; Conway, B.; D'Aquila, R.T.; Demeter, L.M.; Kuritzkes, D.R.; Pillay, D.; Schapiro, J.M.; Telenti, A.; Richman, D.D. Update of the drug resistance mutations in HIV-1: 2004. Top. HIV. Med. **2004**, 12, 119–124.
2. Panel on Clinical Practices for Treatment of HIV Infections, Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. http://aidsinfo.nih.gov/guidelines/adult/archive/AA_102904.pdf (accessed Jan 2005).
3. Turpin, J.A. The next generation of HIV/AIDS drugs: novel and developmental anti-HIV drugs and targets. Expert. Rev. Anti. Infect. Ther. **2003**, 1, 97–128.
4. Broom, A.D.; Agrawal, V.K.; Tutonda, M.G.; Fain, H.D.; Buckheit, R.W. Unusual single-stranded polyribonucleotides as potent anti-HIV agents. J. Med. Chem. **1995**, 38, 3253–3257.

5. Tutonda, M.G.; Fain, H.D.; Buckheit, R.W.; Broom, A.D. The role of hydrophobic versus hydrophilic bases in the anti-HIV activity of purine-containing polyribonucleotides. Nucleosides & Nucleotides **1997**, 16, 173–182.
6. Tutonda, M.G.; Buckheit, R.W.; Agrawal, V.K.; Broom, A.D. Antiviral oligo- and polyribonucleotides containing selected triazolo[2,3-*a*]purines. J. Med. Chem. **1998**, 25, 4958–4964.
7. Hyde, R.M.; Buckheit, R.W.; Broom, A.D. "Senseless" antiviral polyribonucleotides: Poly(1-propargylinosinic acid). Nucleosides, Nucleotides & Nucleic Acids **2002**, 21, 45–54.
8. Hyde, R.M.; Broom, A.D.; Buckheit, R.W. Antiviral amphipathic oligo- and polyribonucleotides: Analogue development and biological studies. J. Med. Chem. **2003**, 46, 1878–1885.
9. Buckheit, R.W., Jr.; Lackman-Smith, C.; Snow, M.J.; Jalliday, S.M.; White, E.L.; Ross, L.J.; Agrawal, V.K.; Broom, A.D. PMTI, a broadly active unusual single-stranded polyribonucleotide inhibits virus replication by multiple mechanisms. Antiviral Chem. Chemother. **1999**, 10, 23–32.
10. Langer, M.; Kratz, F.; Rothen-Rutishauser, B.; Wunderli-Allenspach, H.; Beck-Sickinger, A.G. Novel peptide conjugates for tumor specific chemotherapy. J. Med. Chem. **2001**, 44, 1341–1348.
11. Golden, P.L.; Pardridge, W.M. Brain microvascular P-glycoprotein and a revised model of multidrug resistance in brain. Cell. Mol. Neurobiol. **2000**, 20, 165–181.
12. Uyechi, L.S.; Gange, L.; Thurston, G.; Szoka, R.C., Jr. Mechanism of lipophilic gene deliver in mouse lung: Binding and internalization of fluorescent lipid and DNA components. Gene Ther. **2001**, 8, 828–836.
13. Rudolf, M.P.; Fausch, S.C.; Da Silva, D.M.; Kast, W.M. Human dendritic cells are activated by chimeric human papillomavirus type 16 virus-like particles and induce epitope-specific human T-cell response in vitro. J. Immunol. **2001**, 166, 5917–5924.
14. Witvrouw, M.; Fikkert, V.; Pluymers, W.; Matthews, B.; Mardel, K.; Schols, D.; Raff, J.; Debyser, Z.; De Clercq, E.; Holan, G.; Pannecouque, C. Polyanionic (i.e., polysulfonate) dendrimers can inhibit the replication of human immunodeficiency virus by interfering with both virus adsorption and later steps (reverse transcriptase/integrase) in the virus replication cycle. Mol. Pharm. **2000**, 58, 1100–1108.
15. Karczewski, M.K.; Strebel, K. Cytoskeleton association and virion incorporation of the human immunodeficiency virus type 1 Vif protein. J. Virol. **1996**, 70, 494–507.
16. Ugolini, S.; Moulare, M.; Mondor, I.; Barois, N.; Demandolx, D.; Hoxike, J.; Brelot, A.; Alizon, M.; Davoust, J.; Sattentau, Q.J. HIV-1 gp120 induces an association between CD4 and the chemokine receptor CXCR4. J. Immunol. **1997**, 159, 3000–3008.
17. Stauber, R.H.; Rulong, S.; Palm, G.; Tarasova, N.I. Direct visualization of HIV-1entry: Mechanisms and role of cell surface response. Biochem. Biophys. Res. Commun. **1999**, 258, 695–702.
18. Hermida-Matsumoto, L.; Resh, M.D. Localization of human immunodeficiency virus type 1 Gag and Env at the plasma membrane by confocal imaging. J. Virol. **2000**, 74, 8670–8679.
19. Sandefur, S.; Smith, R.A.; Varthakavi, V.; Spearman, P. Mapping and characterization of the N-terminal I domain of human immunodeficiency virus type 1. J. Virol. **2000**, 74, 7238–7249.
20. Chen H.; Boyle, T.J.; Malim, M.H.; Cullen, B.R.; Lyerly, H.K. Derivation of a biologically contained replication system for human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. U.S.A. **1992**, 89, 7678–7682.
21. Kiser, R.; Makovsky, S.; Terpening, S.J.; Laing, N.; Clanton, D.J. Assessment of a cytoprotection assay for the discovery and evaluation of anti-human immunodeficiency virus compounds utilizing a genetically impaired virus. J. Virol. Meth. **1996**, 58, 99–109.
22. Because the emission of fluorescence for the 50 and 1000 nM samples demonstrated the same pattern of increasing fluorescent intensity over time, those images are not included.
23. Pompon, A.; Lefebvre, I.; Imbach, J.-L. "On-line internal surface reversed-phase cleaning": The direct HPLC analysis of crude biological samples. Application to the kinetics of degradation of oligonucleotides in cell culture medium. Biochem. Pharmacol. **1992**, 8, 1769–1775.
24. Data not shown.
25. The 1A2 cell line was maintained by Heidi Fain in the Pharmacology & Toxicology Department, University of Utah. The confocal microscopy data was acquired in collaboration with Keith Jensen, Pharmaceutics and Pharmaceutical Chemistry, University of Utah.
26. Equilibration buffer and fixing reagent from Molecular Probes, Eugene, Oregon.