Pergamon

Bioorganic & Medicinal Chemistry 9 (2001) 2165–2169

BIOORGANIC & MEDICINAL CHEMISTRY

Anti-HIV-1 Activity of an Antisense Phosphorothioate Oligonucleotide Bearing Imidazole and Primary Amine Groups

Kaoru Ushijima,[a] Masahiro Shirakawa,[a] Koumei Kagoshima,[a] Wee-Sung Park,[a] Naoko Miyano-Kurosaki[b] and Hiroshi Takaku[a,b,*]

[a]*Department of Industrial Chemistry, Chiba Institute of Technology, 2-17-1 Tsudanuma, Narashino, Chiba 275-0016, Japan*
[b]*High Technology Research Center, Chiba Institute of Technology, 2-17-1 Tsudanuma, Narashino, Chiba 275-0016, Japan*

Received 13 February 2001; accepted 14 April 2001

Abstract—We have previously shown that RNA cleaving reagents with imidazole and primary amine groups on the 5′-end of antisense oligodeoxyribonucleotides could site-specifically cleave CpA as the target sequence of the substrate tRNA in vitro. In this study, a RNA cleaving reagent, composed of imidazole and primary amine groups on an antisense phosphorothioate oligonucleotide (Im-anti-s-ODN), was synthesized and evaluated for anti-HIV-1 activity in MT-4 cells. The sequence of the Im-anti-s-ODN was designed to be complementary to the HIV-1 *gag*-mRNA and to bind adjacent to the CpA cleavage site position. Im-anti-s-ODN encapsulated with the transfection reagent, DMRIE-C®, had higher anti-HIV-1 activity than the unmodified antisense phosphorothioate oligonucleotide (anti-s-ODN) at a 2 μM concentration. Furthermore, the Im-anti-ODN encapsulated with DMRIE-C® conferred sequence-specific inhibition.

Introduction

Antisense oligonucleotides have been used successfully to inhibit gene expression in a number of biological systems.[1–3] This paradigm of gene regulation has many potential therapeutic applications. Advances in the understanding of the function, metabolism, and structure of these molecules have led to the development of antisense molecules with enhanced nuclease resistance and increased specificity, selectivity, and potency.[4]

Antisense regulation or attenuation of protein synthesis can be applied to any candidate gene with a known molecular sequence. Antisense molecules are short strands of DNA or RNA, usually 16–20 bases in length, that are synthesized to complement a target region of a candidate gene. The antisense molecule binds to its complementary region, and through a number of mechanisms, inhibits or attenuates gene expression. The success of antisense oligonucleotides in the inhibition of protein synthesis in a number of biological systems presents a new approach to gene therapy and protein regulation.[5]

Alternatively, a number of investigators have focused on the development of synthetic ribonuclease mimics (artificial ribonucleases) that can be covalently attached to an oligonucleotide and thereby promote a cleavage reaction on its RNA target.[3,4] Artificial ribonucleases are expected to become useful tools for molecular biology and in the development of new anti-viral or anti-cancer therapeutics.

Artificial ribonucleases fall into two categories; those that function by general acid/base catalysis, mimicking enzymes, and those that make use of metal coordination complexes by a nucleophilic reaction. The former includes polypeptides,[6–9] oligoamines,[10–13] and imidazole conjugates of intercalating agents,[14–17] while the latter encompasses transition–metal ions[18] and transition–metal complexes.[19–22]

An approach to the design of such molecules could be the imitation of the active centers of ribonucleases. In order to design artificial ribonucleases that possess site-specific cleavage ability, the use of base-specific ribonucleases is more effective. The mechanism of RNase A activity is one of the attractive models.

RNase A contains two essential histidines and one essential lysine in its catalytic center.[23–26] The actions of

*Corresponding author. Tel.: +81-47-478-0407; fax: +81-47-471-8764; e-mail: takaku@ic.it-chiba.ac.jp

0968-0896/01/$ - see front matter
PII: S0968-0896(01)00126-2

this enzyme are relatively well understood, and studies have provided deep insights into the respective mechanisms.[25–28] RNase A cleaves predominantly the pyrimidine-A position on RNA sequences and shows a bell-shaped pH dependence (a maximum at pH 7.0).

We have previously synthesized sequence-specific RNA cleaving molecules by attaching polyamine derivatives bearing imidazole and/or primary amine groups to the 5′-end of antisense oligonucleotides (Im-DNA and PA-DNA), thus mimicking of the active center of RNase A.[29] These cleaving molecules hydrolyzed selectively at the CpA of the target sequences of the substrate tRNA in vitro. In particular, Im-DNA showed higher site-specific cleavage activity than PA-DNA.

In this paper, we synthesized an artificial ribonuclease derivative bearing imidazole and primary amine groups at the 5′-end of the antisense phosphorothioate oligonucleotide (Im-anti-s-ODN), directly adjacent to the complementary target sequence on the *gag* mRNA from HIV-1. We evaluated for the anti-HIV-1 activity of this modified oligonucleotide encapsulated with a transfection reagent (DMRIE-C®) in HIV-1 infected MT-4 cells. This modified oligonucleotide (Im-anti-s-ODN) encapsulated with DMRIE-C® had higher anti-HIV-1 activity than the antisense phosphorothioate oligonucleotide (anti-s-ODN), and showed sequence-specific inhibition.

Results and Discussion

Chemical synthesis

Antisense and random phosphorothioate oligonucleotides bearing imidazole and primary amine groups (Im-anti-s-ODN and Im-ran-s-ODN) were synthesized from *N,N′*-bis-(2-aminoethyl)-*N*-ethyl-1[dimethoxytrityl]-1-H-imidazole-4-acetamide and a phosphorothioate oligodeoxyribonucleotide by solid phase synthesis, as described previously[29–31] (Fig. 1b). The anti-s-ODN was purchased from KURABO Co. Japan. The design of the 20-mer antisense phosphorothioate oligonucleotide bearing imidazole and primary amine groups (Im-anti-s-DNA) is shown in Figure 1b. It is complementary to the sequence (784-803) of the *gag* mRNA from the HIV-1 genome shown in Figure 1a. This antisense sequence is adjacent to the CpA position, which is the predominant cleavage site of the artificial ribonuclease mimicking RNase A.[29] The control 20-mer random sequence phosphorothioate oligonucleotide bearing imidazole and primary amine groups (Im-ran-s-ODN) is also shown in Figure 1c.

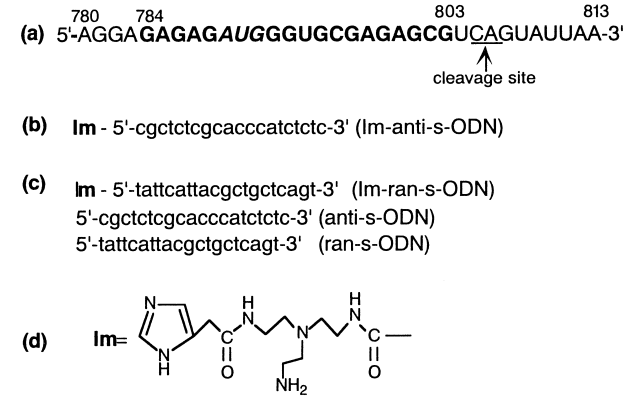

Figure 1. (a) Target site (784-803) of HIV-1 *gag*-sequence containing the *AUG* initiation codon; (b) Imidazole and primary amine groups conjugated at the 5′-end of the HIV-1-antisense phosphorothioate oligonucleotide (Im-anti-s-ODN); (c) The control phosphorothioate oligonucleotides, Im-ran-s-ODN, anti-s-ODN, and ran-s-ODN; (d) **Im** is the moiety composed of imidazole and primary amine groups.

The FITC-labeled-Im-anti-s-ODN and FITC-labeled-anti-s-ODN were prepared from the Im-anti-s-ODN, and the anti-s-ODN, using the Label IT® Nucleic Acids Labeling kit according to the manufacturer's instructions (Mils Co.).

Biological assays

In order to clarify the anti-HIV-1 activities of the modified oligonucleotide (Im-anti-s-ODN), we tested it in MT-4 cells. Previously, we observed that a hairpin antisense oligonucleotide, complementary to the site of the HIV-1 *gag* sequence (770–801) containing the AUG initiation codon, showed inhibitory effects as compared to hairpin antisense oligonucleotides containing the regions of splice acceptors or the AUG initiation codon sites of HIV-1 *rev*, *tat,* and *pol*.[32]

In this study, the modified antisense phosphorothioate oligonucleotide (Im-anti-s-ODN), with an AUG, initiation codon sequence as the target of the HIV-1 *gag*-gene (784-803), was synthesized and tested for anti-HIV-1 activity in MT-4 cells. Control oligonucleotides were prepared for comparison, such as an antisense phosphorothioate oligonucleotide (anti-s-ODN), and also randomly ordered oligonucleotides (Im-ran-s-ODN and ran-s-ODN).

The 50% inhibitory concentration (EC_{50}) for cytopathic effects (CPE) on MT-4 cells induced by HTLV-IIIB and the 50% cytotoxic concentration (CC_{50}) of the modified antisense oligonucleotides were determined by an MTT assay, according to the procedure reported previously.[33,34] The anti-HIV-1 activity of Im-anti-s-ODN is shown in Table 1. The Im-anti-s-ODN possessed an EC_{50} value of 0.32 μM, whereas the control drugs, anti-s-ODN, Im-ran-s-ODN, and AZT, had EC_{50} values of

Table 1. Anti-HIV-1 activities of oligonucleotides and AZT by MTT assay

	EC_{50} (μM)[a]	CC_{50} (μM)[b]
Im-anti-s-ODN	0.32	>100
Im-ran-s-ODN	0.36	68.0
Anti-s-ODN	0.37	>100
AZT	0.07	292.5

[a]Anti-HIV-1 activity, concentration required for 50% inhibition of HIV-1 induced cytopathogenicity in MT-4 cells, as determined by the MTT method.
[b]Cytotoxicity, concentration required for 50% viability in MT-4 cells, as determined by the MTT method.

0.36, 0.37, and 0.073 μM, respectively. No cytotoxicity of Im-anti-s-ODN was observed, even at a 100 μM (CC_{50}) concentration of compound. However, the control oligonucleotide, Im-ran-s-ODN, also protected against HIV-1 induced CPE. These results showed that the Im-anti-s-ODN and anti-s-ODN inhibited HIV-1 induced CPE, but the Im-ran-s-ODN also inhibited HIV-1 replication in HIV-1 infected MT-4 cells. Phosphorothioate oligonucleotides have been shown to block the proliferation of HIV-1 in acutely infected cells in a non-sequence dependent manner,[35–38] probably by the inhibition of reverse transcriptase[39,40] and/or the viral entry process.[41,42]

Antisense oligonucleotide systems have been used as inhibitors of gene expression in various culture systems, and are considered to be potential therapeutic agents against cancer and viral infections. In order to exert any of these effects, the oligonucleotides must enter into the cytoplasm and the nuclear compartment of the cell. Thus, transport and intracellular delivery are important considerations when developing an effective oligonucleotide-based therapy. Liposome delivery is one technique that addresses these concerns. Attempts to use liposomes in the delivery of antisense oligonucleotides have been reported.[43] Cationic lipids are being used for the delivery of oligonucleotides for therapeutic, and research purposes, and are the vehicles of choice for some gene therapy protocols.[44–47]

Accordingly, we investigated the inhibitory effects of the modified antisense oligonucleotide (Im-anti-s-ODN) encapsulated with a transfection reagent. The MT-4 cells were incubated with HTLV-IIIB (moi = 0.01) for 1 h at 0 °C to allow absorption. The cells were then washed to remove the virus from the medium, and the oligonucleotides encapsulated with DMRIE-C® were added with fresh medium. We have tested the transfection efficiencies of antisense phosphorothioate oligonucleotides into the MT-4 cells using commercially available liposomal reagents. Using our experimental setting, the best results were obtained with DMRIE-C® (unpublished data). The virus production in the culture supernatant was monitored by the HIV-1 p24 antigen assay (Fig. 2). The control-infected cells (no oligomer added) exhibited maximal HIV-1 replication at 5 days. However, in the cells treated with the Im-anti-s-ODN (2 μM), p24 expression was greatly inhibited, as compared to the untreated control (Fig. 2). In contrast, the anti-s-ODN inhibited HIV-1 replication at a low level, as compared to the untreated control, after 5 days (Fig. 2). On the other hand, the cells treated with the control random oligomers (Im-ran-s-ODN and ran-s-ODN) expressed high levels of p24 products. These results suggest that the modified antisense oligonucleotide bearing the imidazole group (Im-anti-s-ODN) showed higher anti-HIV activity as compared with the anti-s-ODN. Furthermore, the Im-anti-s-ODN encapsulated with DMRIE-C® conferred sequence-specific inhibition.

The problem in the use of an antisense oligonucleotide is that its cellular uptake is inefficient.[48,49] The use of various enhancers to increase the intracellular accumulation of the antisense phosphorothioate oligonucleotide has largely solved this problem, and has greatly facilitated their use as research tools in vitro. Many cellular uptake enhancers have been reported, including cationic lipids, liposomes, peptides, dendrimers, polycations, cholesterol conjugates, and electroporation. One of the most commonly used enhancers is a mixture of neutral and cationic lipids.[45,50–53]

Efficient cellular uptake of Im-anti-s-ODN is a critical step to enable targeting to the mRNA. We examined the cellular uptake of fluorescently (FITC)-Im-anti-s-ODN encapsulated with DMRIE-C® by treating MT-4 cells with the oligonucleotide for 5 days at 37 °C. As shown in Fig. 3, the FITC intensity of the FITC-Im-anti-s-ODN

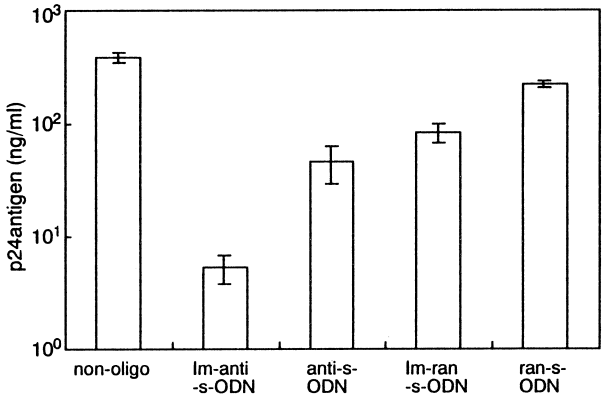

Figure 2. Anti HIV-1 activities of modified oligonucleotides at a concentration of 2 μM. The MT-4 cells were incubated with HTLV-IIIB (moi = 0.01) for 1 h at 0 °C to allow absorption. The cells were then washed to remove the virus from the medium, and the oligonucleotides encapsulated with DMRIE-C® were added with fresh medium. After 5 days, supernatants were collected and p24 expression was determined by the p24 antigen assay.

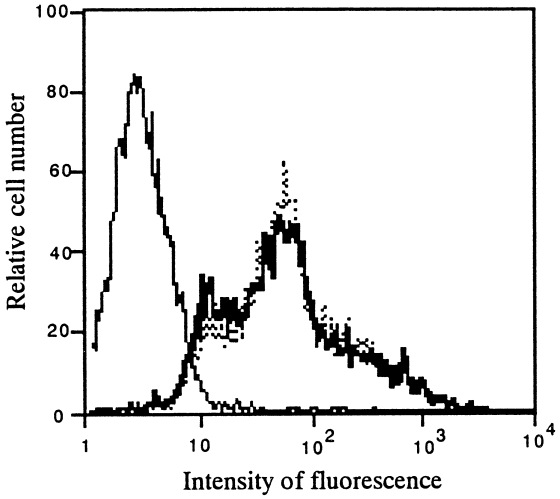

Figure 3. Cellular uptake of the modified and unmodified oligonucleotides encapsulated with DMRIE-C®. The FITC-labeled oligonucleotides (2 μM) encapsulated with DMRIE-C® were incubated with MT-4 cells at 37 °C for 5 days. The dotted-line histogram represents FITC-Im-anti-s-ODN treatment of MT-4 cells. The bold-line histogram represents FITC-anti-s-ODN treatment of MT-4 cells. The thin-line histogram represents the control MT-4 cells in the absence of the FITC-labeled oligonucleotides.

was the same as that of the FITC-labeled-anti-s-ODN. These results suggest that the Im-anti-s-ODN showed very high inhibitory effects as compared to the anti-s-ODN, and the enhanced anti-HIV-1 activity may be due to the presence of the imidazole group.

Conclusion

The antisense phosphorothioate oligonucleotide bearing the 5′-imdazole group (Im-anti-s-ODN) encapsulated with DMRIE-C® inhibited the replication of HIV-1 more effectively than the antisense oligonucleotide (anti-s-ODN), indicating sequence-specific inhibition of HIV-1 replication without the inhibition of reverse transcriptase and/or the viral entry process. The use of the transfection reagent, DMRIE-C®, provides a simple and efficient method for the successful intracellular delivery of the oligonucleotides. The 5′-imidazole group of the antisense phosphorothioate oligonucleotide was found to enhance its anti-HIV-1 efficacy. This type of artificial ribonuclease should be useful as an antiviral agent.

Experimental

Oligonucleotide synthesis

Antisense and random phosphorothioate oligonucleotides bearing imidazole and primary amine groups (Im-anti-s-ODN and Im-ran-s-ODN) were synthesized from *N,N′*-bis-(2-aminoethyl)-*N*-ethyl-1[dimethoxytrityl]-1-H-imidazole-4-acetamide and a phosphorothioate oligodeoxyribonucleotide by solid phase synthesis, as described previously[29,39,40] (Fig. 1b). The anti-s-ODN was purchased from KURABO Co. Japan.

The FITC-labeled-Im-anti-s-ODN and anti-s-ODN were prepared from Im-anti-s-ODN and anti-s-ODN, using the Label IT® Nucleic Acids Labeling kit according to the manufacturer's instructions (Mils Co.).

Cell lines and virus

The human T lymphotropic virus type I (HTLV-I)-positive human T cell line, MT-4, was cultured in RPMI-1640 medium (Gibco BRL Co.) supplemented with 10% FCS (Dainippon Pharmaceutical Co. Ltd.) and 1% penicillin/streptomycin (GIBCO BRL Co.) at 37 °C in a 5% CO_2-gassed incubator.

The HIV-1 strain, HTLV-IIIB, was used for the anti-HIV assay. The virus was prepared from culture supernatants of MOLT-4/HTLV-IIIB cells, which were persistently infected with HTLV-IIIB. The HIV stock solution was titrated in MT-4 cells and was stored at −80 °C until use.

Anti-HIV assay

The anti-HIV activities of the modified oligonucleotides in a cell-free infection were determined for protection against HIV-induced cytopathic effects. MT-4 cells were infected with HTLV-IIIB at a multiplicity of infection (moi) of 0.01. HIV-infected or mock-infected MT-4 cells (25×10^4 cells/mL) were placed into 96-well microtiter plates and were incubated in the presence of various concentrations of the test compounds. All experiments were performed in triplicate. After 5 days of culture at 37 °C in a CO_2 incubator, the cell viability was quantified by a colorimetric assay monitoring the ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to a blue formazan product. Absorbances were read in a microcomputer-controlled photometer (Titertec Multican; Labsystem Oy, Helsinki, Finland) at two wavelengths (540 and 690 nm). The absorbance measured at 690 nm was automatically subtracted from that at 540 nm, to eliminate the effects of non-specific absorption. All data represent the mean values of triplicate wells. These values were then translated into percentages per well, cytotoxicity, and antiviral protection.

MT-4 cells were infected with HTLV-IIIB at an moi of 0.01. HIV-infected or mock-infected MT-4 cells (30×10^4 cells/mL) were placed into 24 well microtiter plates for 1 h at 0 °C. They were washed with RPMI 1640. The oligonucleotide transfection was achieved by combining the modified oligonucleotides (Im-anti-s-ODN, Im-ran-s-ODN, anti-s-ODN, or ran-s-ODN, 2 μM) with 20 μL of DMRIE-C® (2 mg/mL) in RPMI 1640 to achieved a final transfection volume of 500 μL. This solution was then added to infected MT-4 cells (30×10^4 cells). The cells were incubated for 5 h at 37 °C in a 5% CO_2 incubator, and then 500 μL of 20% FCS/RPMI1640 were added. After 5 days, the anti HIV-1 activity was determined by a supernatant p24 antigen measurement using an HIV-1 p24 chemiluminescent enzyme immunoassay (CLEIA Kit, LumiPulse® I, FUJIREBIO INC.).

Cellular uptake of Im-anti-s-ODN encapsulated with DMRIE-C® in MT-4 cells

A mixture of the labeled-modified oligonucleotide (FITC-Im-anti-s-ODN or FITC-anti-s-ODN, 2 μM) and 20 μL of DMRIE-C® (2 mg/mL) in 330 μL of RPMI1640 was added to infected MT-4 cells (30×10^4 cells). After 5 h, 500 μL of 20% FCS/RPMI1640 were added to the cell cultures. After 5 days of culture at 37 °C in a 5% CO_2 incubator, the cells were washed with PBS, resuspended in 1mL of 50% HCOH/PBS, and analyzed by the FACSCalibur® and CellQuest® software (Becton Dickinson, San Jose, CA).

Acknowledgements

This work was supported in part by a Grant-in-Aid for High Technology Research from the Ministry of Education, Science, Sports, and Culture, Japan, and a Research Grant (HIV Grant-K-1031) from the Human Science Foundation.

References and Notes

1. Zamecnic, P. C.; Stephenson, M. L. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 280.
2. Stephenson, M. L.; Zamecnic, P. C. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 285.
3. Crooke, S.T.; Meunier, B. Eds. *Antisense Research and Applications*; CRC Press: Boca Raton, 1993, 330.
4. Baker, B. F.; Monia, B. P. *Biochim. Biophys. Acta* **1999**, *1489*, 3.
5. Stein, C.A.; Krieg, A.M. Eds. *Applied Antisense Oligonucleotide Technology*; Wiley-Liss press: New York, 1998, 173.
6. Zuckermann, R. N.; Schultz, P. G. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 1766.
7. Perello, M.; Barbier, B.; Brack, A. *J. Pept. Protein Res.* **1991**, *38*, 154.
8. Uchiyama, Y.; Inoue, H.; Ohtsuka, E.; Nakai, C.; Ikehara, M. *Bioconjugate Chem.* **1994**, *5*, 327.
9. Perello, M.; Barbier, B.; Brack, A. *Int. J. Pept. Protein Res.* **1991**, *38*, 154.
10. Yoshinari, K.; Yamazaki, K.; Komiyama, M. *J. Am. Chem. Soc.* **1991**, *113*, 5899.
11. Breslow, R.; Bergger, D.; Huang, D.-L. *J. Am. Chem. Soc.* **1990**, *112*, 3686.
12. Komiyama, M.; Inokawa, T. *J. Biochem.* **1994**, *116*, 719.
13. Endo, M.; Azuma, Y.; Soga, Y.; Kuzuza, A.; Kawai, G.; Komiyama, M. *J. Org. Chem.* **1997**, *62*, 846.
14. Hovinen, J.; Guzaev, A.; Azhayeva, E.; Lönnberg, H. J. *J. Org. Chem.* **1995**, *60*, 2205.
15. Reynolds, M. A.; Beck, T. A.; Say, P. B.; Schwartz, D. A.; Dwyer, B. P. *Nucleic Acid Res.* **1996**, *24*, 760.
16. Vlassov, V.; Abramova, T.; Godocikova, T.; Giega, R.; Dilnikov, V. *Antisense Nucleic Acid Drug Dev.* **1997**, *7*, 39.
17. Shinozuka, K.; Shimizu, K.; Nakashima, Y.; Sawai, H. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1979.
18. Breslow, R.; Huang, D.-L. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *88*, 4080.
19. Bashkin, J. K.; Frolova, E. I.; Sampath, U. *J. Am. Chem. Soc.* **1994**, *116*, 5981.
20. Matumura, K.; Endo, M.; Komiyama, M. *J. Chem. Soc., Chem. Commun.* **1994**, 2019.
21. Magda, D.; Miller, R. A.; Sessler, J. L.; Iverson, B. L. *J. Am. Chem. Soc.* **1994**, *116*, 7439.
22. Modak, A. S.; Gard, J. K.; Merriman, M. C.; Winkeler, J. K.; Bashkin, J. K.; Dtern, M. K. *J. Am. Chem. Soc.* **1991**, *113*, 283.
23. Barnard, E. A. *Annu. Rev. Biochem.* **1969**, *38*, 677.
24. Boyer, P.D. Eds.; *The Enzymes*; Academic: New York, 1971; Vol.4.
25. Wyckoff, H. W.; Tsernoglou, D.; Hanson, A. W.; Knox, J. R.; Lee, B.; Richards, F. M. *J. Biol., Chem.* **1970**, *245*, 305.
26. Wlodwar, A.; Bott, R.; Sjölin, L. *J. Biol. Chem.* **1982**, *257*, 1325.
27. Findley, D.; Herries, D. G.; Mathias, A. P.; Rabin, B. R.; Ross, C. A. *Biochem. J.* **1962**, *85*, 152.
28. Deavin, A.; Mathias, A. P.; Rabin, B. R. *Nature* **1966**, *211*, 252.
29. Ushijima, K.; Gouzu, H.; Hosono, K.; Shirakawa, M.; Kagosima, K.; Takai, K.; Takaku, H. *Biochim. Biophys. Acta* **1998**, *1379*, 217.
30. Bashkin, J. K.; Gard, J. K.; Modak, A. S. *J. Org. Chem.* **1990**, *55*, 5125.
31. Bonfils, E.; Depierreux, C.; Midoux, P.; Thoung, N. T.; Monsingly, M.; Roche, A. C. *Nucleic Acids Res.* **1992**, *20*, 4621.
32. Kuwasaki, T.; Hosono, K.; Takai, K.; Ushijima, K.; Nakashima, H.; Saito, T.; Tamamoto, N.; Takaku, H. *Biochem. Biopys. Res. Commun.* **1996**, *228*, 623.
33. Pauwels, R.; Balzarini, T.; Baba, M.; Snoeck, R.; Schols, D.; Herdewijn, P.; Desmyter, J.; De Clercq, E. J. *J. Virol. Method* **1988**, *20*, 309.
34. Nakashima, H.; Pauwels, R.; Baba, M.; Schois, D.; Demyter, J.; De Clrecq, R. *J. Viol. Method* **1989**, *26*, 319.
35. Matsukura, M.; Zon, G.; Shinozuka, K.; Robert-Guroff, M.; Shimada, T.; Stein, C. A.; Mitsuya, H.; Wong-Staal, F.; Cohen, J. S.; Broder, S. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 4244.
36. Stein, C. A.; Matsukura, M.; Subasinghe, C.; Broder, S.; Cohen, J. S. *AIDS Research and Human Retroviruses* **1989**, *5*, 639.
37. Agrawal, S.; Goodchild, J.; Civeira, M. P.; Sarin, P. S.; Zamecnik, P. C. *Nucleosides Nucleotides* **1989**, *8*, 819.
38. Kinchington, D.; Galpin, S.; Jaroszewski, J. W.; Ghosh, K.; Subasinghe, C.; Cohen, J. S. *Antiviral Res.* **1992**, *17*, 53.
39. Majumdar, C.; Stein, C. A.; Cohen, J. S.; Broder, S.; Wilson, S. H. *Biochemistry* **1989**, *23*, 1340.
40. Maury, G.; El Allaoui, A.; Morvan, F.; Müller, B.; Imbach, J.-L.; Goody, R. S. *Biochem. Biopys. Res. Commun.* **1992**, *186*, 1249.
41. Stein, C. A.; Neckers, L. M.; Nair, B. C.; Mumbauer, S.; Hoke, G.; Pal, R. *J. Acquir. Immu. Defic. Syndr.* **1991**, *4*, 686.
42. Hatta, T.; Kim, S.-G.; Nakashima, H.; Yamomoto, N.; Sakamoto, K.; Yokoyama, S.; Takaku, H. *FEBS Lett.* **1993**, *330*, 161.
43. Bennett, M. R.; Anglin, S.; McEwan, J. R.; Jagoe, R.; Newby, A. C.; Evan, G. I. *J. Clin. Invest.* **1994**, *93*, 820.
44. Zhu, N.; Liggitt, D.; Lui, Y.; Debs, R. *Science* **1993**, *261*, 209.
45. Felgner, J. H.; Kumer, R.; Sridhar, C. N.; Wheeler, K.; Tsai, Y. J.; Border, R.; Ramsey, P.; Martin, M.; Felgner, P. L. *J. Biol. Chem.* **1994**, *269*, 2550.
46. Logan, J. L.; Bebok, Z.; Walker, L. C.; Peng, S.; Felgner, P.; Siegal, G. P.; Frizzell, R. A.; Dong, J.; Howard, M.; Matalon, S.; Lindsay, J. R.; Duvall, M.; Sorcher, E. J. *Gene Therapy* **1995**, *2*, 38.
47. Walker, L.; Irwin, W. J.; Akhtar, S. *Pharmacol. Res.* **1995**, *12*, 1548.
48. Leserman, L.; Machy, P.; Leonetti, J. P.; Milhaud, P. G.; Degols, S. G.; Lebleu, B. *Prog. Clin. Biol. Res.* **1990**, *343*, 95.
49. Akhtar, S.; Juliano, R. L. *Trends Cell Biol.* **1992**, *2*, 139.
50. Lasic, D. D.; Papahadjopoulos, D. *Science* **1995**, *267*, 1275.
51. Bennett, C. F.; Chiang, M. Y.; Chang, H.; Shoemaker, J. E.; Mirabelli, C. K. *Mol. Pharmacol.* **1992**, *41*, 1023.
52. Zhou, X.; Huang, L. *Biochim. Biophys. Acta* **1994**, *1189*, 195.
53. Lewis, J. G.; Lin, K. Y.; Kothavale, A.; Flanagan, W. M.; Matteucci, M.; DePrince, R. B.; Mook, R. A.; Hendren, R. W., Jr; Wagner, R. W. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 3176.