Biochemical Pharmacology, Vol. 43, No. 12, pp. 2581–2589, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

INHIBITION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 REVERSE TRANSCRIPTASE BY 3′-BLOCKED OLIGONUCLEOTIDE PRIMERS

SABINE AUSTERMANN,* MOGENS KRUHØFFER* and FRANK GROSSE†
Department of Virology and Immunology, German Primate Centre, Kellnerweg 4, W-3400 Göttingen, Germany

(*Received* 12 *November* 1991; *accepted* 9 *March* 1992)

Abstract—Human immunodefficiency virus type 1 (HIV-1) reverse transcriptase (RT) (EC 2.7.7.49) with a high specific activity has been purified from the overexpressing *Escherichia coli* strain DH5α[pJS3.7]. Steady-state kinetics of DNA synthesis catalysed by RT were analysed on polyriboadenylate 20-mer of (3′-5′)deoxythymidylate [poly(rA)·$(dT)_{20}$] and polyribouridylate 20-mer of (3′-5′)-deoxyadenylate [poly(rU)·$(dA)_{20}$] homopolymeric template–primers. K_m values of 40 and 140 nM (3′-OH ends) and k_{cat} values of 4 and 0.14 sec^{-1} were determined for the two different substrates. Oligonucleotide primers $(dA)_{20}$ and $(dT)_{20}$ were elongated in a terminal transferase-catalysed reaction (EC 2.7.7.31) with ddATP, 3′-dATP (cordycepin), 2′,3′-epoxy-ATP and arabino-ATP; and ddTTP, 3′-azido-TTP, 3′-dUTP, 3′-F-dTTP and rUTP, respectively. The resulting oligonucleotides were hybridized to their complementary templates and the inhibitory potential of these compounds towards DNA synthesis started from unchanged primers was measured. Oligonucleotides with unextendable 3′-groups were shown to act as strong inhibitors of DNA synthesis catalysed by HIV-1 RT. In particular, poly(rA)·$(dT)_{20}$-[ddTMP] and poly(rU)·$(dA)_{20}$-[3′-dAMP] were potent competitive inhibitors, displaying K_i values of about 6 and 12 nM, respectively. Also 3′-azido-, and 3′-fluoro-terminated oligonucleotides showed competitive inhibition with inhibition constants in the range of 20–35 nM. In contrast, 2′,3′-epoxy-terminated $(dA)_{21}$ displayed a mixed-type inhibition with a K_i value of 67 nM. Arabino-terminated $(dA)_{21}$ was found to be an uncompetitive inhibitor of HIV-1 RT with an inhibition constant of 318 nM. Arabino-terminated primers did not act as strict chain terminators because they could be elongated by HIV-1 RT. This study provides information on the structure–activity relationship of modified 3′-termini of primer molecules which might be exploited as inhibitors of HIV in the future.

Human immunodeficiency virus 1 (HIV-1‡) is the causative agent of acquired immunodeficiency syndrome (AIDS) [1–3]. The HIV reverse transcriptase (RT) (EC 2.7.7.49) is responsible for replication of the viral genome. The enzyme catalyses both RNA-directed DNA synthesis and DNA-directed DNA synthesis in the infected host cell. Since the RT is crucial for viral growth and such an activity apparently does not exist in non-infected human cells, it has become the main target for antiviral therapies [4]. 3′-Azido-2′,3′-dideoxyribosylthymidine (AZT, zidovudine) is the most prominent representative of a class of nucleoside analogs which inhibits the replication of HIV-1 and is currently used therapeutically [4, 5]. Two other drugs, 2′,3′-dideoxycytidine and 2′,3′-dideoxyinosine, have shown promising results in therapeutic approaches [6]. These nucleoside analogs are intracellularly phosphorylated to the corresponding 5′-triphosphates. The triphosphate analogs exert their inhibitory potential towards HIV replication by acting as competitive inhibitors of the pool of naturally occurring deoxynucleoside triphosphates [7]. Because the intracellular phosphorylation steps are rather inefficient [8, 9], relatively high amounts of these drugs must be prescribed in order to achieve intracellular concentrations that are sufficient to inhibit HIV replication. This in turn has led to serious side-effects [10], which are thought to be caused by an interaction of the analog triphosphates with cellular DNA polymerases.

In recent studies on the inhibition of HIV-1 RT 3′-azido-2′,3′-dideoxyribosylthymidine 5′-triphosphate (AZTTP) it was shown that AZTTP is not only a competitive inhibitor of dTTP but also acts as an alternate substrate which becomes incorporated into a growing nucleotide chain [11, 12]. After incorporation, the resulting 3′-end is no longer extendable by HIV RT and thus acts as a strong and competitive inhibitor of unchanged 3′-OH group-bearing primers [11, 12]. This type of inhibitor might gain in therapeutic interest because of the very low K_i values, the obvious circumvention of the

* Present address: Niedersachsisches Institut für Peptidforschung, Feodor-Lynen-Strasse 31, W-3000 Hannover 61, F.R.G.

† Corresponding author. Tel. (49) 551-3899296; FAX (49) 551-3899388.

‡ Abbreviations: AIDS, acquired immunodeficiency syndrome; arabino-ATP, adenine-9-β-D-arabino-furanoside 5′-triphosphate; AZT, 3′-azido-2′,3′-dideoxyribosylthymidine; AZTTP, 3′-azido-2′,3′-dideoxyribosylthymidine 5′-triphosphate; $(dA)_{20}$, 20-mer of (3′-5′)deoxyadenylate; $(dT)_{20}$, 20-mer of (3′-5′)deoxythymidylate; 3′-F-dTTP, 3′-fluoro-3′deoxythymidine 5′-triphosphate; HIV-1, human immunodeficiency virus type 1; KP_i, potassium phosphate buffer; oligo(dA), oligomer of deoxyadenylate; oligo(dT), oligomer of deoxythymidylate; poly(rA), polyriboadenylate; poly(rI), polyriboinosylate; poly(rU), polyribouridylate; RT, reverse transcriptase.

intracellular phosphorylation steps and last but not least the possibility of specifically directing these inhibitors to the HIV genome by using oligonucleotide sequences that are complementary to the retroviral RNA. As a first step in evaluating the inhibitory potential of 3′-blocked oligonucleotides we have synthesized oligonucleotides with various non-extendable blocking groups at their 3′-termini and analysed their inhibotory potential towards DNA synthesis catalysed by HIV-1 RT.

MATERIALS AND METHODS

Materials. ddATP, ddTTP, rUTP, 3′-dUTP, 3′-dATP and adenine-9-β-D-arabine-furanoside 5′-triphosphate (arabino-ATP) were purchased from Pharmacia (Freiburg, F.R.G.). AZTTP, 3′-fluoro-3′-deoxythymidine 5′-triphosphate (3′-F-dTTP) and 2′,3′-epoxy-ATP were synthesized by the method of Ludwig and Eckstein [13] and kindly provided by Prof. F. Eckstein, Max-Planck-Institute for Experimental Medicine, Göttingen, F.R.G. dTTP, dATP, rUTP, polyribouridylate [poly(rU)] and polyriboadenylate [poly(rA)] were purchased from Boehringer-Mannheim (F.R.G.) and 20-mer of (3′,5′)deoxyadenylate [$(dA)_{20}$] was supplied by Pharmacia. 20-mer of (3′,5′)deoxythymidylate [$(dT)_{20}$] was synthesized on a 380B DNA synthesizer from Applied Biosystems by the phosphoroamidite method. [γ-^{32}P]ATP and [α-^{32}P]dNTPs (3000 Ci/mmol) were from Amersham-Buchler (Braunschweig, F.R.G.). All other chemicals were of analytical grade.

Terminal deoxynucleotidyl transferase (EC 2.7.7.31;17.4 U/mL) and T4 polynucleotide kinase (EC 2.7.1.78; 10 U/mL) were obtained from Stratagene (Heidelberg, F.R.G.).

Radioactive labeling of oligonucleotides. Radioactive labeling of the 5′-ends was performed with T4 polynucleotide kinase as described previously [14]. The labeled oligonucleotides were separated from radioactive ATP by using Sep-Pak™ cartridges (Waters Associates, Milford, MA, U.S.A.).

PAGE. The purity of 5′-labeled oligonucleotides was analysed on 20% polyacrylamide gels containing 8 M urea [15]. Samples were mixed with stop-solution (95% formamide, 20 mM EDTA, 0.05% Bromophenol blue, 0.05% xylenccyanol), heated to 95° for 2 min and applied to the gel. 5′-^{32}P-Labeled $p(dT)_{20}$ and $p(dT)_{10}$ served as length standards. After 3.5 hr of electrophoresis at 25 V/cm, gels were autoradiographed for 12 hr with Cronex 4-films at −80°.

Preparation of template–primers. Polymeric templates and oligomeric primers were mixed in a molar ratio (nucleotide) of 2.5 to 1 in water. The solutions were heated for 2 min at 70° and then slowly cooled down to room temperature. The solutions were stored at −20°.

Enzyme assays. Unless otherwise indicated, RT was assayed as described previously [16]. Inhibition reaction were typically carried out in a volume of 50 μL. The reaction mixture consisted of 50 mM Tris–HCl, pH 8.0, 80 mM KCl, 6 mM $MgCl_2$, 1 mM dithiothreitol, 0–80 nM 3′-OH-ends of unmodified primer annealed to the complementary template, 0–100 nM modified primer annealed to its complementary template, 50 μM [^{3}H]dTTP (108 cpm/pmol) and [α-^{32}P]dATP (256 cpm/pmol), and enzyme in an amount that ensured initial rate conditions. All reactions were carried out at 37°. After 2.5, 5, 7.5 and 10 min incubation at 37°, portions of 10 μL were spotted onto GF 34-filters (Schleicher & Schüll, Dassel, F.R.G.) and passed into ice-cold 10% trichloroacetic acid. After 5 min, filters proceeded to a suction device and were washed 10 times with 2 mL 1 M HCl (each) and five times with 1 mL 95% ethanol, dried and counted in a 1211 Minibeta (LKB, Bromma, Sweden) liquid scintillation counter. DNA synthesis was quantified by applying a least-squares fitting procedure to the data points of the incorporation kinetics and evaluating the corresponding slopes.

Purification of HIV RT. Escherichia coli DH5α bacteria were freshly transformed with the recombinant plasmid [pJS3.7] containing the open reading frame of the complete HIV-1 *pol* gene fused to the β-galactosidase gene and a collagen gene linker [16, 17]. Cells were grown in LB medium containing 50 mg/L ampicillin at 32° until an optical density at 600 nm of 1 was obtained. Then, 1 mg/L isopropyl-β-D-thiogalactoside was added to induce the production of the β-galactosidase–collagen–*pol* fusion protein, which is cleaved in an autocatalytic reaction by its internal protease domain [16]. Five hours after induction, the cells were harvested by centrifugation (20 min, 2500 *g*) and resuspended in 1/20 of their original volume of 50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 3 mM β-mercaptoethanol, 100 μg/mL lysozyme. Opening of the cells was completed by sonication and cellular debris was removed by centrifugation (30 min, 20,000 *g*). To one volume of the resulting crude extract one (wet) volume of DEAE-cellulose (DE52, Whatman), equilibrated with buffer A (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 3 mM β-mercaptoethanol) was added and the slurry was stirred for 30 min at 4°. Unbound proteins were removed by suction through a glass-sintered Buchner funnel and subsequent washing with three column volumes of buffer A. More than 80% of the RT activity was found in the unbound fraction. This material was directly loaded onto a second DEAE-cellulose column (1 v/v crude extract, equilibrated with buffer A). Typically, less than 10% of the activity was found in the flow-through of this column. The column was washed with three bed volumes buffer A and then step-eluted with three bed volumes buffer A containing 0.2 M NaCl. Active fractions were combined, the pH was titrated to 7.0 and the conductivity was adjusted to that of buffer B (50 mM Tris–HCl, pH 7.0, 1 mM EDTA, 3 mM β-mercaptoethanol, 100 mM NaCl). This solution was then loaded onto a phosphocellulose column (0.02 bed vol./mL crude extract) equilibrated with buffer B. The column was washed with three bed volumes of buffer B and eluted with a linear gradient (10 bed volumes) from 0 to 0.5 M NaCl in buffer B. RT-containing fraction were collected and mixed with hydroxyapatite (0.002 column vol./mL crude extract), equilibrated in buffer A (20 mM KP_i, pH 6.8, 1 mM dithiothreitol) for 30 min and then decanted into a column. A linear

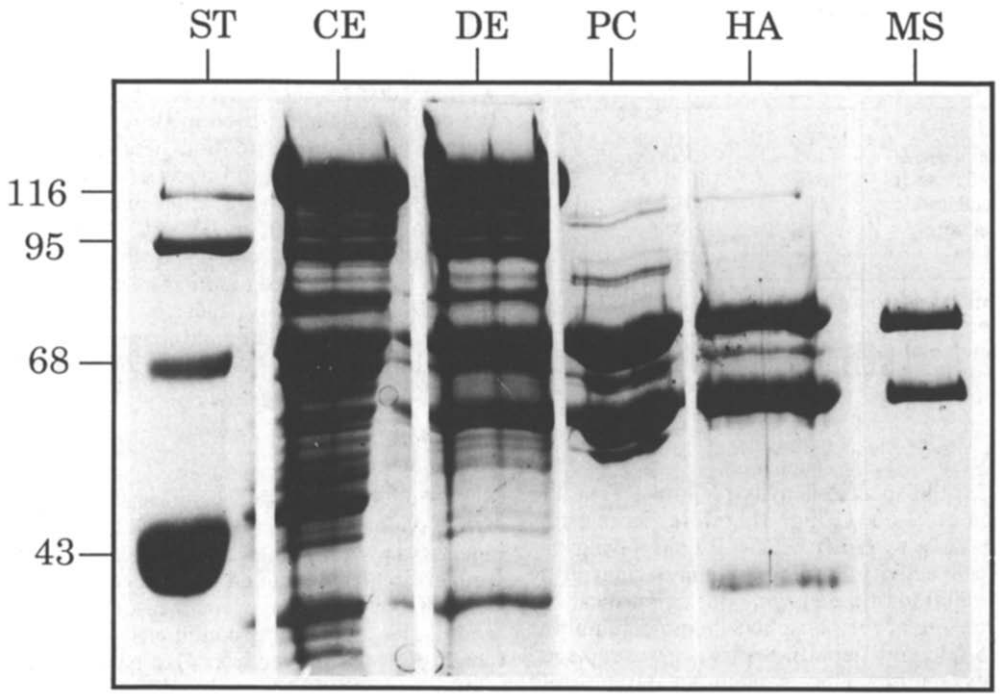

Fig. 1. SDS–PAGE of recombinant HIV-1 RT at various stages of purification. Fractions containing 3–10 μg protein were prepared for electrophoresis by precipitation with 10% trichloroacetic acid. Separation was on a 10% SDS gel. After electrophoresis, the gel was stained with Coomassie blue. CE, crude bacterial extract; DE, second DEAE cellulose eluate; PC, phosphocellulose eluate; HA, hydroxyapatite eluate; MS, FPLC Mono S eluate. Molecular mass standards (ST) were β-galactosidase (116 kDa), phosphorylase b (95 kDa), bovine albumin (68 kDa), and ovalbumin (43 kDa).

gradient (10 column volumes) from 20 to 120 mM potassium phosphate, pH 6.8, was used for elution. Fractions with RT activity were combined and dialysed against buffer A for 4 hr. Final purification was achieved by Mono S chromatography, which had been equilibrated with buffer A. A gradient from 0 to 200 mM KCl eluted the RT at about 120 mM KCl. The final fraction was >95% pure. On SDS–PAGE it displayed two polypeptide bands of approximately equal intensities at 66 and 51 kDa (Fig. 1). Specific activity of the purified enzyme was 20,000 U/mg. One unit (U) of RT activity is the amount of enzyme that catalyses the incorporation of 1 nmol dTMP in 10 min at 37° into poly(rA)·oligo(dT)$_{20}$ [16].

Synthesis of 3′-modified oligonucleotides with terminal deoxynucleotidyl transferase. The reaction solution contained in a total volume of 300 μL either 100 mM potassium cacodylate, pH 7.2, 0.2 mM dithiothreitol, 1 mM $MgCl_2$ (for purine insertions) or $CoCl_2$ (for pyrimidine insertions), 10% ethylene glycol or, alternatively, 20 mM Tris acetate, pH 7.2, 0.2 mM dithiothreitol, 1 mM $MgCl_2$. Oligomer of deoxythymidylate [oligo(dT)] was used at a concentration of 7.5 μM (3′-ends) and oligomer of deoxyadenylate [oligo(dA)] at a concentration of 25 μM (3′-ends); furthermore, 0.5 mM of the modified nucleoside triphosphates and 30–200 U terminal transferase were used. After incubation for between 1 and 20 hr at 37° a portion was withdrawn, labeled with [γ-^{32}P]ATP and analysed by denaturing PAGE. After the times given in Table 2, the reaction mixture was loaded onto a preparative DuPont 850 Liquid Chromatograph. the stationary phase was ODS Hypersil (5 μm; Shandon Inc., Runcorn, U.K.) in a 1 × 25 cm column, the flow rate was 1.5 mL/min. A linear gradient from 3.5 to 56% acetonitrile in 50 mM triethylammonium bicarbonate pH 7.5 was applied for 30 min. Product containing fractions were collected, evaporated to dryness, and the residual material was re-evaporated with ethanol to remove traces of buffer. After labeling with [γ-^{32}P]ATP, the product was analysed and compared to [5′-^{32}P]-p(dT)$_{20}$ by denaturing PAGE. A single band with the expected mobility and a purity of >95% was obtained and used for further experiments.

RESULTS

Preparation of highly active recombinant HIV-1 RT

RT isolated from virus particles [18, 19] or from recombinant bacteria consists of a mixture of a 66- and a 51-kDa polypeptide (p66 and p51), in the latter case, however, only if the protease domain has been co-expressed in the bacteria [16, 20–22].

Recently, we reported on the purification of HIV-1 RT from the recombinant *E. coli* strain JM109 [JS3.7]. This procedure gave heterodimeric RT with a specific activity of 4000 U/mg on poly(rA)·oligo(dT) [16]. However, based on a turnover number of

Table 1. Purification of bacterially expressed HIV-1 RT

Fraction	Protein (mg)	Specific activity (U/mg)	Yield (%)
Crude extract	1239	31	—
DEAE cellulose I	730	330	100
DEAE cellulose II	189	1000	78
Phosphocellulose	21	5000	43
Hydroxyapatite	9	9900	37
FPLC Mono S	4	20,000	33

Purification was from 10 g of *E. coli* DH5α[pJS3.7]. RT activity could not be quantified in crude extracts because of the presence of nuclease.

4 sec^{-1} [23] the specific activity of pure RT should be as high as 21,000 U/mg. Therefore, more effort has been made to purify HIV-1 RT displaying this high specific activity. This was mainly achieved by avoiding dilution of the enzyme during purification. Hence, the size of the phosphocellulose column was reduced 5-fold, and heparin-Sepharose was replaced by hydroxyapatite. This avoided an earlier used dilution step and allowed a further concentration of the enzyme, because the size of the hydroxyapatite column was only 1/50 the size of the heparin-Sepharose. Final purification to an enzyme with >95% homogeneity was achieved by chromatography on an FPLC Mono S column. An outline of the different purification steps is shown in Fig. 1 and Table 1. It should be noted that by using this procedure it was no longer necessary to include detergents or glycerol to protect the RT activity.

Characterization of HIV-1 RT by steady-state kinetics

The initial rate of DNA synthesis performed by HIV-1 RT was measured on either poly(rA)·$(dT)_{20}$ or poly(rU)·$(dA)_{20}$ template–primers with dTTP or dATP as the nucleotide substrate. By employing both Eadie–Hofstee (not shown) and Lineweaver–Burk evaluations (Fig. 2) [24], the K_m values for 3′-OH primer ends were measured to be 40 ± 5 (N = 3) and 140 ± 20 nM (N = 3), respectively. The values for the catalytic rate constant were derived from the corresponding V_{max} values by considering one active binding site and 100% active enzyme to be 4 ± 1 sec^{-1} and 0.14 ± 0.06 sec^{-1} (N = 3) on poly(rA)·$(dT)_{20}$ and poly(rU)·$(dA)_{20}$, respectively (see legend for Fig. 2).

Elongation of oligonucleotides with nucleotide analogs by terminal deoxynucleotidyl transferase

Oligonucleotide primers were blocked at their 3′-termini by adding non-extendable nucleotide analogs. The addition was carried out enzymatically by using the terminal deoxynucleotide transferase reaction. It was observed that reaction conditions for the elongation varied dramatically for the different analogs used. Most efficient elongation was observed with ddATP. In a reaction volume of 300 μL 30 U of terminal transferase and an incubation time of 2 hr were sufficient to elongate more than 99% of 25 μmol (3′-ends) primer molecules. In contrast, with 3′-deoxy, 3′-fluoro-, 3′-azido-modified nucleotide analogs and UTP, incubation times of up to 20 hr and a 10-fold higher amount of terminal transferase were needed for an elongation of only 50–70% of the primers in the reaction mixture (Table 2). The course of primer extension was controlled by analysing the reaction products on polyacrylamide gels after different incubation times. After the reaction times shown in Table 2, products were taken and purified from unelongated primers by HPLC. HPLC-purified products were again analysed by gel electrophoresis, then hybridized to their complementary templates and subsequently used for inhibition studies on purified recombinant HIV-1 RT.

Inhibition of HIV-1 RT by 3′-modified oligonucleotides

The influence of 3′-blocked primer termini on the DNA synthesis reaction of HIV RT was measured against varying amounts of the homologous template–primers, which contained unchanged 3′-OH primer termini. All reactions were measured in triplicate and under initial rate conditions. The inhibition constants were derived from Dixon plots ($1/v$ vs [I]) (Fig. 3 and Table 3). Since it is difficult to distinguish between competitive and mixed-type inhibition by means of Dixon plots, the type of inhibition was derived from a Lineweaver–Burk evaluation of the data. Most of the blocked oligonucleotides displayed a competitive type of inhibition when hybridized to their corresponding templates, as expected for substrate-like analogs (Table 3). The inhibition constants varied between 6 and 36 nM, dependent on the blocking group and the template–primer system used (Fig. 3). Dideoxy-blocked $(dT)_{21}$ (Fig. 3A) was more inhibitory than 3′-azido-blocked (Fig. 3B), followed by 3′-deoxyuridylate- (Fig. 3C) and 3′fluorothymidylate-blocked primers (Fig. 3D). Poly(rA)·$(dT)_{20}$-$(rU)_2$ served as an alternate substrate and had, as expected, no inhibitory effect on the elongation reaction of poly(rA)·$(dT)_{20}$ catalysed by HIV-1 RT (Fig. 3E). With blocked $(dA)_{21}$ primers, 3′-deoxyadenylate (cordycepin) (Fig. 3F) and dideoxyadenylate (Fig. 3G) were most effective, followed by 2′,3′-epoxy- (Fig. 3H) and arabino-terminated primers (Fig. 3I). The Lineweaver–Burk evaluation of the inhibition data for 2′,3′-epoxy-terminated primers revealed a mixed-type inhibition rather than a pure linear competitive inhibition with a K_i value of 67 nM (Table 3). Finally, the arabino-modified oligonucleotide displayed an uncompetitive type of inhibition with an inhibition constant of 318 nM (Fig. 3I). The inhibition data are summarized in Table 3.

Arabino-AMP-terminated primers are extendable by HIV-1 RT

The uncompetitive mode of inhibition and the rather weak inhibition constant observed with poly(rU)·$(dA)_{20}$-[arabino-AMP] prompted us to analyse in a more detailed way, whether HIV RT can elongate a primer terminus carrying an arabino instead of the normal ribo- or deoxyribo configuration. For this purpose we elongated poly(rU)·$(dA)_{20}$ with arabino-ATP to produce

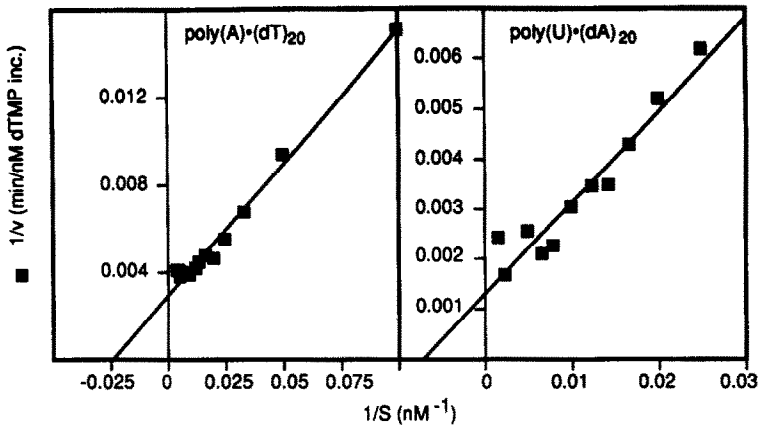

Fig. 2. Determination of the Michaelis constants for poly(rA)·(dT)$_{20}$ and poly(rU)·(dA)$_{20}$. The incorporation of [^{3}H]dTMP and [α-^{32}P]dAMP was determined on varying amounts of poly(rA)·(dT)$_{20}$, respectively. The reaction was started by the addition of RT to 50 μL of prewarmed (37°) reaction mixture. Concentration of dNTPs were 100 μM; RT was added to final concentrations of 1.45 nM for dTMP incorporation (left panel) and 89.9 nM for dAMP incorporation (right panel). The corresponding V_{max} values can be taken from the intersection with the ordinate as 345 nM/min (left panel) and 760 nM/min (right panel). From these values and the corresponding concentrations of enzyme used, k_{cat} values of 4 sec^{-1} for synthesis on poly(rA)·(dT)$_{20}$ and of 0.14 sec^{-1} for synthesis on poly(rU)·(dA)$_{20}$ can be calculated by using the relationship $k_{cat} = V_{max}/[RT]\cdot 60$. The factor of 60 is necessary for expressing k_{cat} in terms of sec^{-1} instead of min^{-1}.

Table 2. Reaction conditions for the elongation of the oligonucleotides by various nucleotide analogs

Substrate	Terminal transferase (U)	Incubation time (hr)
ddTTP	30	2
AZTTP*	200	20
3′-F-dTTP*	200	20
UTP	50	16
3′-dUTP*	30	16
ddATP	30	2
3′-dATP*	30	16
2′,3′-Epoxy ATP	30	2
Arabino-ATP	60	2

The elongation reaction were either performed in Tris acetate or, when marked with an asterisk *, in cacodylate buffer as described in Materials and Methods. Mg^{2+} served as a cofactor for the incorporation of modified purine nucleotides; modified pyrimidines were inserted in the presence of Co^{2+}.

substantial amounts of poly(rU)·(dA)$_{20}$-[arabino-AMP] (Fig. 4, lane 2) and also some poly(rU)·(dA)$_{20}$-[arabino-AMP]$_2$ (Fig. 4, lane 3). These results demonstrate that HIV RT is able to accept arabino-ATP as a substrate and to elongate arabino-terminated ends with a second arabino-ATP. The further elongation of arabino-AMP-terminated primers became more apparent when dATP was added to the reaction mixture. In this case, the (dA)$_{20}$[arabino-AMP] band disappeared almost completely and longer product bands showed up (Fig. 4, lane 4).

DISCUSSION

This report provides quantitative data on the inhibitory potential of a novel type of HIV RT inhibitor, i.e. oligonucleotides with unextendable 3′-OH groups. This type of inhibitor can arise from the incorporation of nucleotide analogs into a growing polynucleotide chain [11, 12] and thus may in part be responsible for the inhibition reaction observed with the corresponding nucleoside triphosphate. Thus, quantifying the inhibitory potential of this kind of oligonucleotide may lead to a better understanding of the reaction underlying the inhibition of reverse transcription. Furthermore, this approach may also provide further clues to the development of novel antiviral drugs.

Eight nucleotides with sugar modifications at the 2′ or 3′ hydroxyl group were added enzymatically to corresponding oligonucleotides by using a terminal transferase-catalysed reaction. With the exception of the arabino-terminated primer (see below), all primer termini were found to be unextendable by HIV RT. The inhibitory potential of the resulting primers was analysed by employing steady-state kinetics in the HIV RT-catalysed DNA synthesis reaction. For all 3′-blocked primers K_i values between 6 and 70 nM (modified 3′-ends) were determined; the type of inhibition was in almost all cases competitive against the corresponding primer without a modified 3′-end. In parallel, K_m values for unchanged poly(rA)·(dT)$_{20}$ and poly(rU)·(dA)$_{20}$ were measured to be 40 and 140 nM (primer 3′-ends), respectively. Thus, in the case of both blocked poly(rA)·(dT)$_{20}$ and blocked poly(rU)·(dA)$_{20}$ template–primers, the K_m/K_i varied between 1 and 12. This variation probably reflects the discriminatory

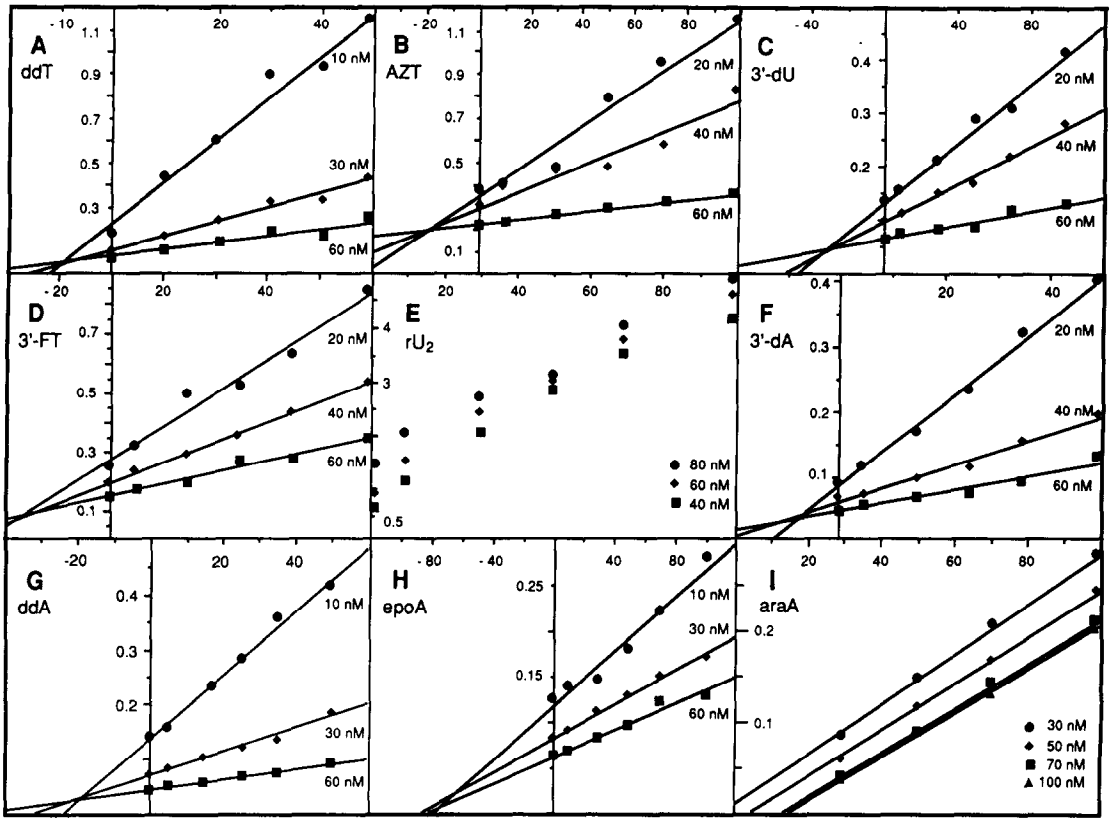

Fig. 3. Inhibition of HIV-1 RT by template–primers containing blocked 3′ OH-termini. Reaction conditions are described in Materials and Methods. Values at the abscissa of each graph indicate the inhibitor concentration [I] in terms of nM blocked 3′-OH ends. The inhibitors used were: panel A, poly(rA)·$(dT)_{20}$-[ddTMP]; panel B, poly(rA)·$(dT)_{20}$-[3′-N_3-TMP]; panel C, poly(rA)·$(dT)_{20}$-[3′-dUMP]; panel D, poly(rA)·$(dT)_{20}$-[3′-F-TMP]; panel E, poly(rA)·$(dT)_{20}$-$[rUMP]_2$; panel F, poly(U)·$(dA)_{20}$-[3′-dAMP]; panel G, poly(U)·$(dA)_{20}$-[ddAMP]; panel H, poly(U)·$(dA)_{20}$-[2′,3′-epoxy-AMP]; panel I, poly(U)·$(dA)_{20}$-[arabino-AMP]. Values at the ordinate are expressed as $1/V$ in minutes per micromolar dNMP incorporated. For all measurements three different concentrations of either poly(rA)·$(dT)_{20}$ (panels A–E) or poly(rU)·$(dA)_{20}$ (panels F–I) have been used as indicated at the right-hand side of each graph.

Table 3. Inhibition of DNA synthesis performed by HIV-1 RT by oligonucleotide primers with 3′-blocked termini

Template–primer	K_i* (nm)	K_i/K_m†	Type of inhibition‡
Poly(rA)·$(dT)_{20}$-[ddTMP]	6	0.146	Competitive
Poly(rA)·$(dT)_{20}$-[3′-N_3-TMP]	19	0.463	Competitive
Poly(rA)·$(dT)_{20}$-[3′-dUMP]	35	0.854	Competitive
Poly(rA)·$(dT)_{20}$-[3′-F-TMP]	36	0.878	Competitive
Poly(rA)·$(dT)_{20}$-$[rUMP]_2$	—	—	No inhibition
Poly(U)·$(dA)_{20}$-[3′-dAMP]	12	0.086	Competitive
Poly(U)·$(dA)_{20}$-[ddAMP]	18	0.129	Competitive
Poly(U)·$(dA)_{20}$-[2′,3′-epoxy-AMP]	67	0.479	Mixed type
Poly(U)·$(dA)_{20}$-[arabino-AMP]	318	2.271	Uncompetitive

* K_i values were taken from Dixon plots as shown in Fig. 3.

† K_m values of 40 nM and 140 nM have been measured for synthesis on poly(rA)·$(dT)_{20}$ and poly(rU)0$(dA)_{20}$, respectively (see legend for Fig. 2 and text).

‡ The type of inhibition was determined by using a Lineweaver–Burk evaluation of the inhibition data.

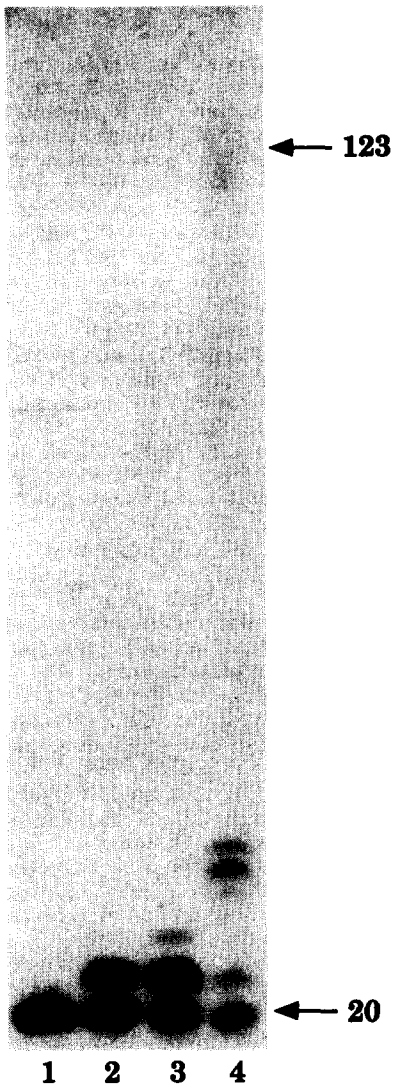

Fig. 4. Incorporation of arabino-ATP into poly(rU)·oligo(dA) and subsequent elongation with dATP catalysed by the RT from HIV-1. One micromolar (nucleotide) 5′-^{32}P-labeled $(dA)_{20}$ hybridized to 110 μM (nucleotide) poly(rU) (lane 1) was incubated in the standard reaction mixture with 0.48 μM HIV RT in the presence of 400 μM arabino-ATP for 1 (lane 2) and 2 hr. After 2 hr of incubation, dATP was added to a final concentration of 600 μM and a sample was withdrawn after another 2 hr of incubation (lane 4). The reaction products were analysed on a 20% polyacrylamide gel containing 8 M urea as described elsewhere [15].

potential of HIV RT against the modification of the 3′-end. Dideoxy- and 3′-deoxy-terminated primers were the most potent inhibitors observed, indicating that the 3′-OH group at the primer terminus, although essential for chain elongation, is probably not recognized by HIV RT as an essential part of the primer. The replacement of 3′-H by either a 3′-F or a 3′-N_3 group seems to reduce the recognition capacity of HIV RT by factors of 3 and 6.

Blockage of oligo(dA) with 2′,3′-epoxy-AMP resulted in a mixed-type mode of inhibition against poly(rU)·oligo(dA). This situation may arise when the enzyme–inhibitor complex has a lower affinity than the enzyme to the template–primer substrate. Alternatively, part of the enzyme molecules might become inactivated by forming a covalent complex with the inhibitor, e.g. via an opening of the reactive 2′,3′-epoxy ring. Such a "suicidal" mode of inhibition has been suggested for the inhibition of *E. coli* DNA polymerase I (EC 2.7.7.7) and the RT from avian myeloma virus by 2′,3′-epoxy-ATP [25]. On the other hand, neither Abboud *et al.* [25] nor Catalano *et al.* [26] were able to detect DNA polymerase I covalently bound to epoxy-terminated primers by means of SDS–PAGE. Like these authors, we failed to detect an incorporation of radioactively labeled $p(dA)_{20}$-[2′,3′-epoxy-AMP] into HIV RT using SDS–PAGE (data not shown). Hence, there is no evidence for a covalent bond between HIV RT and an oligonucleotide-bound 2′,3′-epoxy-AMP.

A further unexpected result was the weak and uncompetitive inhibition of the HIV RT reaction by 3′-arabino-terminated oligo(rA). Uncompetitive inhibition points to a mode of action in which the inhibitor binds to an alternate site of the enzyme–substrate complex changing both the V_{max} and the K_m values. Such a different binding site may be represented by the binding domain of the RNase H activity of HIV RT. However, experiments in order to show whether or not 3′-arabino-terminated oligo(rA) has an influence on the RNase H reaction were negative. This is in agreement with recent studies on the deadenylation of poly(rA)·oligo(dT) by the RNase H activity of HIV-1 RT, from which only one binding site for both enzymatic activities, RNase H and RT, has been inferred [27]. A further analysis of DNA synthesis in the presence of arabino-ATP revealed that HIV RT accepts arabino-ATP as an alternate substrate and thereby produces 3′-arabino-terminated oligo(rA) primers. Poly(rU)·oligo(rA)-arabino-AMP is in a turn a substrate for HIV RT for both the incorporation of dATP and arabino-ATP. Thus, HIV RT accepts the arabino configuration at both nucleoside triphosphates and at the 3′-end of template–primers, though with a reduced incorporation rate. Similar results have been presented recently for the cellular replicase, i.e. the DNA polymerase α-primase complex. The primase activity incorporates arabino-ATP into RNA-primers of newly formed Okazaki fragments. The resulting arabino-terminated RNA-primers are still elongatable by DNA polymerase α [28]. Thus, the arabino group seems not to act as a strict chain terminator for various RNA and DNA polymerases but rather represents a kinetic hindrance for the incorporation of a further nucleotide. Once an arabino-AMP-terminated primer has been elongated by only one more dAMP, the ongoing chain elongation reaction will probably occur with normal kinetics. However, it seems unlikely to us that the described kinetic behavior of arabino-AMP-terminated primers is responsible for the observed uncompetitive mode of inhibition. Thus, the reason(s) for uncompetitive and mixed-type inhibition displayed by some of the template–primers

are not yet understood. In this respect it seems noteworthy that uncompetitive inhibition of HIV RT has also been observed with unprimed poly(rC) [23].

Oligonucleotides have been shown to inhibit potently HIV replication, in both a sequence-specific [29] and a sequence-unspecific manner [30]. The stability of oligonucleotides can be improved by chemical modifications, such as the introduction of phosphorothioates, methylphosphonates or phosphoramidates. However, at least some of the modifications seem to hinder the cellular uptake of oligonucleotides. Once the oligonucleotides have reached the intracellular milieu, three possible modes of action can be discussed: (i) inhibition of translation, (ii) inhibition of transcription and (iii) induction of cellular and/or retroviral RNase H activities, which in turn degrade that part of the viral RNA genome which is hybridized to the oligonucleotide (for recent reviews see Refs 31 and 32). The non-sequence-specific type of inhibition, as exemplified by phosphorothioate-containing $(dC)_{28}$, is probably based on the linear competitive binding of this oligonucleotide to the HIV RT primer binding site [33]. Thus, the antiretroviral effect of oligonucleotides is caused by different effects that superimpose each other.

The introduction of unextendable 3′-termini to oligonucleotides adds some further advantageous features to this type of HIV inhibitor. As shown here, many 3′-blocked oligonucleotides are strong and competitive inhibitors for the RT, displaying K_i values of between 6 and 36 nM. Inhibition of the reverse transcription step is an aspect that is normally not considered to contribute to the molecular effects of antisense oligonucleotides. Furthermore, it was recently demonstrated that 3′-extensions with AZTMP inhibit the 3′ to 5′ exonucleases of the DNA polymerases δ and ε considerably [34]. Similarly, ddCMP-terminated primers were not degraded by the corresponding exonuclease of DNA polymerase γ [35]. From this, it is conceivable that other cellular and extracellular 3′ to 5′ exonucleases might be inhibited as well. If this should turn out to be the case, a higher stability of the terminated oligonucleotides is expected. Hence, combining common antisense approaches, including modifications to improve the penetration through membranes, with the strong inhibitory potential of blocked 3′-termini might be beneficial to the efficacy of this kind of antiviral compound.

Acknowledgements—We are particularly grateful to O. Heidenreich (MPI Exp. Med., Göttingen) for stimulating discussions and continuous help during this work. We thank F. Eckstein (MPI Exp. Med., Göttingen) for the generous supply of 3′-F-dTTP, 2′,3′-epoxy-ATP and AZTTP and F. Benseler (MPI Exp. Med., Göttingen) for synthesizing $(dT)_{20}$. This work was supported by the grant FKIII-003-89 of the *Bundesministerium für Forschung und Technologie*. M. K. appreciates support from the Danish Natural Science Research Council. This work was started at the Max-Planck-Institute for Experimental Medicine. The hospitality of this institute is gratefully acknowledged.

REFERENCES

1. Barré-Sinoussi F, Cherman JC, Rey R, Nugeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin C, Vezinet-Brun F, Rouzioux C, Rozenbaum W and Montagnier L, Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* **220**: 868–871, 1983.
2. Gallo RC, Salahuddin SZ, Popovic M, Shearer GM, Kaplan M, Haynes BF, Palker TJ, Redfield R, Oleske J, Safai B, White G, Foster P and Markham PD, Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science* **224**: 500–503, 1984.
3. Popovic M, Sarngadharan MG, Read E and Gallo RC, Detection, isolation, and continuous production of cytopathic retrovirus (HTLV-III) from patients with AIDS and pre-AIDS. *Science* **224**: 497–500, 1984.
4. Yarchoan R, Mitsuya H, Myers CE and Broder S, Clinical pharmacology of 3′-azido-2′,3′-dideoxythymidine (Zidovudine) and related dideoxynucleosides. *N Engl J Med* **321**: 726–738, 1989.
5. Mitsuya H, Werinhold KJ, Furman PA, St. Clair MH, Nusinoff Lehrman S, Gallo RC, Bolognesi D, Barry DW and Broder S, 3′-Azido-3′deoxythymidine (BW A509U): An antiviral agent that inhibits the infectivity and cytopathic effect of human T lymphotropic virus type III/lymphadenopathy-associated virus *in vitro*. *Proc Natl Acad Sci USA* **82**: 7096–7100, 1985.
6. Broder S, Pharmacodynamics of 2′,3′-dideoxycytidine: an inhibitor of human immunodeficiency virus. *Am J Med* **88** (Suppl 5B): 5B2S–5B7S, 1990.
7. Furman PA, Fyfe JA, St. Clair MH, Weinhold K, Rideout JL, Freeman GA, Nusinoff-Lehrmann S, Bolognesi DP, Broder S, Mitsuya H and Barry DW, Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of 5′-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc Natl Acad Sci USA* **83**: 8333–8337, 1986.
8. Avramis VI, Markson W, Jakson RL and Gomperts E, Biochemical pharmacology of zidovudine in human T-lymphoblastoid cells (CEM). *AIDS* **3**: 417–422, 1989.
9. Balzarini J, Herdewijn P and De Clercq E, Differential patterns of intracellular metabolism of 2′,3′-didehydro-2′,3′-di-deoxythymidine and 3′-azido-2′,3′-dideoxythymidine, two potent anti-human immunodeficiency virus compounds. *J Biol Chem* **264**: 6127–6133, 1989.
10. Fischl MA, Richman DD, Grieco MH, Gottlieb MS, Volberding PA, Laskin OL, Groopman JE, Mildman D, Schooley RT, Jackson GG, Durack DT and King D, The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. *N Engl J Med* **317**: 185–191, 1987.
11. St. Clair MH, Richards CA, Spector T, Weinhold KJ, Miller WH, Langlois AH and Furman PA, 3′-Azido-3′-deoxythymidine triphosphate as an inhibitor and substrate of purified human immunodeficiency virus reverse transcriptase. *Antimicrob Agents Chemother* **31**; 1972–1977, 1987.
12. Heidenreich O, Kruhøffer M, Grosse F and Eckstein F, Inhibition of human immunodeficiency virus reverse transcriptase by 3′-azidothymidine triphosphate. *Eur J Biochem* **192**: 621–625, 1990.
13. Ludwig J and Eckstein F, Rapid and efficient synthesis of nucleoside 5′-*O*-(1-thiotriphosphates), 5′-triphosphates and 2′,3′-cyclophosphorothioates using 2-chloro-4*H*-1,3,2-benzodioxaphophorin-4-one. *J Org Chem* **54**: 631–635, 1989.
14. Maniatis T, Fritsch EF and Sambrook J, *Molecular Cloning—A Laboratory Manual*. Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1982.
15. Hohn KT and Grosse F, Processivity of the DNA

polymerase α primase complex from calf thymus. *Biochemistry* **26**: 2870–2878, 1987.
16. Weber J and Grosse F, Fidelity of human immunodeficiency virus type 1 reverse transcriptase in copying natural DNA. *Nucleic Acids Res* **17**: 1379–1394, 1989.
17. Scholtissek S and Grosse F, A plasmid vector system for the expression of a triprotein consisting of β-galactosidase, a collagenase recognition site and a foreign gene product. *Gene* **62**: 55–64, 1988.
18. Lightfoote MM, Coligan JE, Folks TM, Fauci AS, Martin MA and Venkatesan S, Structural characterization of reverse transcriptase and endonuclease polypeptides of the acquired immunodeficiency syndrome retrovirus. *J Virol* **60**: 771–775, 1986.
19. Di Marzo Veronese F, Copeland TD, DeVico AL, Rahman R, Oroszlan S, Gallo RC and Sarngadharan MG, Characterization of the highly immunogenic p66/p51 as the reverse transcriptase of HTLV-III/LAV. *Science* **231**: 1289–1291, 1986.
20. Hansen J, Schulze T and Moelling K, RNase H activity associated with bacterially expressed reverse transcriptase of human T-cell lymphotropic virus III/lymphadenopathy-associated virus. *J Biol Chem* **262**: 12393–12396, 1987.
21. Le Grice SFJ, Beuck V and Mous J, Expression of biologically active human T-cell lymphotropic virus type III reverse transcriptase in *Bacillus subtilis*. *Gene* **55**: 95–103, 1987.
22. Le Grice SFJ and Grüninger-Leitch F, Rapid purification of homodimer and heterodimer HIV-1 reverse transcriptase by metal chelate affinity chromatography. *Eur J Biochem* **187**: 307–314, 1990.
23. Majumdar C, Abbotts J, Broder S and Wilson SH, Studies on the mechanism of human immunodeficiency virus reverse transcriptase: steady-state kinetics, processivity, and polynucleotide inhibition. *J Biol Chem* **263**: 15657–15665, 1988.
24. Lineweaver H and Burk D, The determination of enzyme dissociation constants. *J Am Chem Soc* **56**: 658–666, 1934.
25. Abboud MM, Sim WJ, Loeb LA and Mildvan AS, Apparent suicidal inactivation of DNA polymerase by adenosine 2′,3′-riboepoxide 5′-triphosphate. *J Biol Chem* **253**: 3415–3421, 1978.
26. Catalano CE and Benkovic SJ, Inactivation of DNA polymerase I (Klenow fragment) by adenosine 2′,3′-epoxide 5′-triphosphate: evidence for the formation of a tight-binding inhibitor. *Biochemistry* **28**: 4374–4382, 1989.
27. Krug MS and Berger SL, Reverse transcriptase from human immunodeficiency virus; a single template–primer binding site serves two physically separable catalytic functions. *Biochemistry* **30**: 10614–10623, 1991.
28. Kuchta RD and Willhelm L, Inhibition of DNA primase by 9-β-D-arabinofuranosyladenosine triphosphate. *Biochemistry* **30**: 797–803, 1991.
29. Agrawal S, Goodchild J, Civeira MP, Thornton AT, Sarin PS and Zamecnik PC, Oligodeoxynucleoside phosphoroamidates and phosphorothioates as inhibitors of human immunodeficiency virus. *Proc Natl Acad Sci USA* 7079–7083, 1988.
30. Matsukura M, Shinizuka K, Zon G, Mitsuya H, Reitz M, Cohen JS and Broder S, Phosphorothioate analogs of oligodeoxynucleotides: inhibitors of replication and cytopatic effects of human immunodeficiency virus. *Proc Natl Acad Sci USA* **84**: 7706–7710, 1987.
31. Englisch U and Gauss DH, Chemically modified oligonucleotides as probes and inhibitors. Angew Chem Int Ed Engl **30**: 613–629, 1991.
32. Uhlmann E and Peyman A, Antisense oligonucleotides: a new therapeutic principle. *Chem Rev* **90**: 543–583, 1991.
33. Majumdar C, Stein CA, Cohen JS, Broder S and Wilson SH, Stepwise mechanism of HIV reverse transcriptase: primer function of phosphorothioate oligodeoxynucleotides. *Biochemistry* **28**: 1340–1346, 1989.
34. Nickel W, Austermann S, Bailek G and Grosse F, Interactions of azidothymidine triphosphate with the cellular DNA polymerases α, δ, and ε and with DNA primase. *J Biol Chem* **267**: 848–854, 1992.
35. Longley MJ and Mosbaugh DW, Properties of the 3′ to 5′ exonuclease associated with porcine liver DNA polymerase γ. Substrate specificity, product analysis, inhibition and kinetics of terminal excision. *J Biol Chem* **266**: 24702–24711, 1991.