From
Microbiology and Tumorbiology Center
Karolinska Institute
Stockholm
Sweden

Reverse transcriptase assays for analysis of resistance to anti-HIV drugs and their mechanism of action

Xingwu Shao

Stockholm 2003

X. W. Shao: Reverse transcriptase assays for analysis of resistance to anti-HIV drugs and their mechanism of action.
Karolinska Institute, Stockholm, 2003

This work was partially sponsored by Cavidi Tech AB, Uppsala, Sweden.

Printed by Kopieringshuset AB, Uppsala, Sweden.

ISBN 91-7349-489-5

To my family

ABSTRACT

Reverse transcriptase (RT) is a viral enzyme and one of the main targets for drugs against human immunodeficiency virus (HIV). This work presents a set of sensitive non-radioactive RT assays were developed: standard RT inhibition assay (IC assay); RT binding inhibition assay; RT-protein detection assay and template-primer destruction assay or chain termination assay (CT assay). All are based on immobilized polyriboadenylic acid [poly(rA)] template in a 96-well microtitre plate, and use oligodeoxythymidylic acid (odT) as primer and 5-bromodeoxyuridine 5´-triphosphate (BrdUTP) as substrate. The incorporated BrdU binds alkaline phosphatase conjugated anti-BrdU antibody that can be quantified by colorimetry or fluorimetry.

A set of substances with known mechanism of action were examined in the RT assays. Different types of inhibitors exhibited different behaviors in relation to their mode of action. The combined use of the assays was able to define which specific step of reverse transcription the inhibitor affected. The IC_{50} values obtained were similar to those reported for soluble RT assays, which indicated that the assays can be used for screening of RT inhibitory substances. The study of four non-nucleoside RT inhibitors (NNRTIs) 9-Cl-TIBO, Nevirapine, MSA-300 and Delavirdine showed that none of them prevented RT binding to template-primer. MSA-300 had a higher affinity for RT than the other NNRTIs tested.

The biochemical mechanism of resistance to AZT remained a puzzle for a long period. The CT assay and a modified IC assay that were supplemented with physiological concentration of GTP were applied for characterization of the RTs from 18 HIV-1 isolates with various susceptibilities to AZT in cell culture. Up to 9-fold and 600-fold variations in susceptibility to AZT-TP were revealed in IC assay and CT assay, respectively. The CT_{50} values had a tendency to increase with the occurrence of increased numbers of mutations associated with AZT resistance. The substitution T39A was observed in two isolates that were highly resistant to primer termination.

An RT purification procedure was developed to quantify and characterize RT from plasma. The procedure consists of three steps: pre-treatment of plasma to inactivate cellular enzymes; immobilizing virions on a gel and removing antiviral drugs, RT blocking antibodies by a wash; lysis of the immobilized virions and elution of viral RT. A study of 391 samples from HIV infected individuals showed a strong correlation between RT load and RNA copies obtained by PCR. The RT load assay could detect a broad range of different HIV subtypes.

Two phenotypic drug (NNRTIs and thymidine analogue drug) susceptibility tests were developed and used for characterization of RT derived directly from plasma. A high degree of concordance was found between the drug susceptibility profiles of plasma RTs and the occurrence of mutations associated with drug resistance. The assays are technically simple, rapid, and do not require complex interpretation of the results.

In conclusion, the presented non-radioactive RT assays proved useful for screening RT inhibitory substances, for dissection of the mode of action of RT inhibitors, and for characterization of drug susceptibility of RTs from various sources.

Key words: HIV-1, RT, antiviral drugs, NRTIs, NNRTIs, drug resistance, viral load

ISBN 91-7349-489-5

Original papers and manuscripts

This thesis is based on the following communications, which will be referred to by their Roman numerals:

I. **Shao X**, Ekstrand DHL, Bhikhabhai R, Källander CFR and Gronowitz JS. (1997) A non-radioactive microtitre plate reverse transcriptase (RT) assay, based on immobilized template, for screening of RT activity inhibitors and evaluation of their mode of action. Antivir Chem Chemother, 8(2): 149-159.

II. **Shao X**, Rytting A-S, Ekstrand DHL, Vrang L, Källander CFR and Gronowitz JS. (1998) Colorimetric assays for evaluation of the mode of action of human immunodeficency virus type 1 non-nucleoside reverse transcriptase inhibitors. Antivir Chem Chemother, 9(2): 167-176.

III. **Shao XW**, Hjalmarsson S, Lennerstrand J, Svennerholm B, Blomberg J, Källander CFR and Gronowitz JS. (2002) Application of a colorimetric chain termination assay for characterisation of reverse transcriptase from 3'-azido-2'3'-deoxythymidine-resistant HIV isolates. Biotechnol. Appl. Biochem., 35:155-164.

IV. Malmsten A, **Shao XW**, Aperia K, Corrigan GE, Sandström E, Källander CF, Leitner T, and Gronowitz JS. HIV-1 viral load determination based on reverse transcriptase activity recovered from human plasma. Submitted manuscript.

V. **Shao XW**, Malmsten A, Lennerstrand J, Sönnerborg A, Unge T, Gronowitz JS and Källander CFR. (2003) Use of HIV-1 reverse transcriptase recovered from human plasma for phenotypic drug susceptibility testing. AIDS, in print.

ABBREVIATIONS

3TC	lamivudine
aa	amino acid
ABC	abacavir
Ab	antibody
AIDS	acquired immunodeficiency syndrome
AP	alkaline phosphatase
ATP	adenosine 5'-triphosphate
AZT	3´-azido-2´-deoxythymidine
AZT-TP	3´-azido-2´-deoxythymidine triphosphate
BIC_{50}	inhibitor concentration that blocks enzyme binding to template-primer complex by 50%
BrdU	5-bromo-2´-deoxyuridine
BrdUTP	5-bromo-2´-deoxyuridine triphosphate
CD4	cell surface determinant 4
cDNA	complementary DNA
CT_{50}	inhibitor concentration giving 50% residual primer function
ddC	zalcitibine
ddI	didanosine
ddN	dideoxynucleoside
ddNMP	dideoxynucleoside monophosphate
ddNTP	dideoxynucleoside triphosphate
ddTTP	dideoxythymidine triphosphate
DLV	delavirdine
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dTTP (TTP)	deoxythymidine triphosphate
ED_{50}	drug dose giving 50 % reduction of virus replication in cell culture
EFV	efavirenz
ELISA	enzyme linked immunosorbent assay
HAART	highly active antiretroviral therapy
HIV (-1/2)	human immunodeficiency virus (type 1 or 2)
IC_{50}	inhibitor concentration giving 50% inhibition of enzyme activity
Ig	immunoglobulin
Km	Michaelis constant
MAb	monoclonal antibody
mRNA	messenger RNA
MUP	4-methylumbelliferyl phosphate
NEMs	nucleotide excision mutations
NNRTIs	non nucleoside reverse transcriptase inhibitors
NRTI	nucleoside reverse transcriptase inhibitors
NVP	nevirapine
odT	oligodeoxythymidylic acid

p(dA)	polydeoxyadenylic acid
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PFA	foscarnet
PI	protease inhibitor
pNPP	para nitrophenyl phosphate
poly(rA)	polyriboadenylic acid
poly(rG)	polyriboguanylic acid
poly(U)	polyuridylic acid
RNA	ribonucleic acid
RNase H	ribonuclease H
RT	reverse transcriptase
TDC_{50}	inhibitor concentration that destroys 50% of the template-primer function
TDF	tenofovir disoproxil fumarate

CONTENTS

1 INTRODUCTION

In the late 1970s and early 1980s, previously healthy patients with symptoms of immunologic dysfunction sought advice and treatment from physicians in the United States and Europe. This new and unusual syndrome was characterized by generalized lymphadenopathy, opportunistic infections, and a variety of unusual cancers (non-Hodgkin's lymphoma and Kaposi's sarcoma). Many of these individuals had unusually low numbers of CD4+ T-lymphocytes. These symptoms defined a new disease: the acquired immunodeficiency syndrome (AIDS). The causative agent of AIDS was isolated first by Barré-Sinoussie et al in 1983 and Gallo et al in 1984. It was then called lymphadenopathy-associated virus (LAV) and human T-cell leukemia virus III (HTLV III), respectively. Later the virus was renamed human immunodeficiency virus type 1 (HIV-1) (Coffin et al., 1986).

In 1986, an additional HIV, called HIV-2, was isolated (Clavel et al., 1986) from a patient in West Africa, where this virus is dominant. HIV-2 gives a disease similar to HIV-1 but with a lower pathogenic potential for heterosexual spread (Kanki et al., 1994).

HIV is transmitted by direct sexual contact, either homosexual or heterosexual; by blood or blood products; and from an infected mother to infant, either intrapartum, perinatally, or via breast milk (Fauci and Lane, 1991). More than 60 million people have been infected with HIV since the epidemic began and 22 million have died. In 2002, the AIDS epidemic claimed more than 3 million lives, and an estimated 5 million people acquired HIV—bringing the number of people globally living with the virus to 42 million (UNAIDS, 2002). Of the global total of people who are living with HIV, 95% live in developing countries. As the epidemic evolves further, the rate will continue to rise in communities and nations where poverty, social inequalities, and weak health infrastructures facilitate spread of the virus. HIV/AIDS is the leading cause of death in sub-Saharan Africa and the fourth biggest killer in the world.

Sixteen anti-HIV drugs have been approved for treatment of HIV infection. Highly active antiretroviral (ARV) therapy (HAART) has led to a sharp decline in AIDS-related morbidity and mortality. Yet treatment failure is still common. Furthermore, treatment is so far mainly limited to those who can afford it. There is a great need to develop more selective drugs that do not cause rapid development of resistance and have less side effects, as well as cheap and simple methods for measuring viral load and drug resistance.

2 GENERAL VIROLOGY OF HIV-1

2.1 Virion structure and genomic organization of HIV-1

The HIV is a roughly spherical, enveloped virus with a diameter of approximately 100 nm. The virion structure is schematically represented in Fig. 1. The virus genome consists of two copies of positive strand RNA. The RNA is located in an inner viral core (CA, p24, capsid protein) of the virus particle together with reverse transcriptase (RT), protease (PR), integrase (IN) and nucleocapsid proteins (NC). The cone-shaped capsid is encapsulated in a sphere of

matrix protein (MA), coated with a lipid bilayer acquired from the cell membrane as the virion is budding from the host cell. In the lipid bilayer, trimers of the transmembrane glycoprotein (TM, gp41) are anchored, and to this protein the external envelope glycoprotein (SU, gp120) is non-covalently bound. Gp120 contains the determinants that interact with receptor and coreceptor, while gp41 not only anchors the gp120/gp41 complex in the membrane, but also contains domains that are critical for catalyzing the membrane fusion reaction between viral and host lipid bilayers during virus entry.

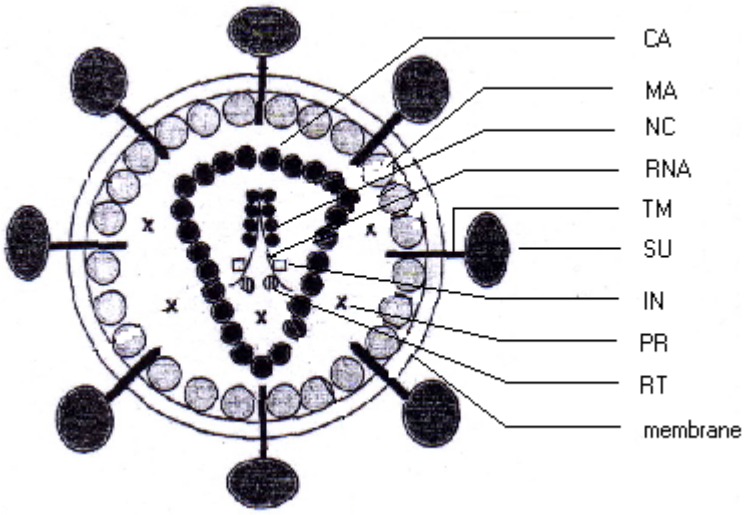

Figure 1. Schematic structure of an HIV-1 virion (adapted from Freed, 1998).

The HIV-1 genome is approximately 9.2 kb long and consists of two viral structural genes (gag and env), a gene for enzymatic proteins (pol), and six regulatory and accessory genes (vif, vpr, vpu, nef, tat and rev) that are flanked by long terminal repeat sequences (LTR), present at both ends of the viral RNA genome (Fig.2). The LTRs encode numerous binding sites for cellular transcription factors. The 5'-LTR contains a cellular tRNAlys annealing site which serves viral RNA inhibitions (Barat et al., 1989), whereas the 3'-LTR regulates viral RNA transcriptional termination.

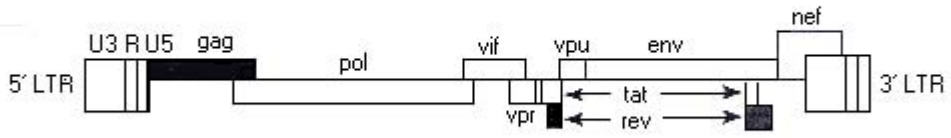

Figure 2. Genomic organization of HIV-1.

The gag gene encodes the precursor for virion capsid proteins that make up the inner nucleoside core. The pol gene encodes the viral enzymatic machinery such as RT, PR and IN. The env gene encodes the precursor for the envelope glycoprotein. Six accessory proteins participate in the viral transcriptional regulatory activities influencing infection efficiency and viral release (Greene, 1991; Trono, 1995; Vaishnav and Wong-Staal, 1991). Tat is critical for transcription from the HIV-1 LTR and Rev plays a major role in the transport of viral RNAs from the nucleus to the cytoplasm. vpu, vif, vpr and nef have been termed "accessory" or

"auxiliary" proteins to reflect the fact that they are not uniformly required for virus replication.

2.2 HIV-1 classification

HIV-1 can be classified into subtypes based on phylogenetic analysis of *gag* and env gene sequences. Three major groups have been defined to classify the circulating HIV-1 strains: M (main), O (outlier) and N (non-M or O) (Gurtler et al., 1994; Kostrikis et al., 1995; Louwagie et al., 1993; Nkengasong et al., 1993; Vanden Haesevelde et al., 1994). The M group of HIV-1, which includes over 95% of the global virus isolates, consists of at least eight discrete clades (A, B, C, D, F, G, H, and J), based on the sequence of complete viral genomes (Louwagie et al., 1993; Myers, 1994). All of the HIV-1 group M subtypes can be found in Africa, while clade B remains the most prevalent isolates in Europe and North America. Clade C accounts for 48% of HIV-1 infections worldwide (Novitsky et al., 1999). Group O is a relatively rare group. Their genomes share less than 50% similarity in nucleotide sequence with group M virus (Subbarao and Schochetman, 1996). A somewhat variable immunodominant gp41 region accounts for a 20% failure rate in identifying HIV-1 group O infected individuals by enzyme-linked immunosorbent assay (ELISA), capable of detecting virtually all infections with HIV-1 group M isolates. Unlike group M viruses, group O strains do not require cyclophilin A for biologic activity (Braaten et al., 1996). The most recently discovered group N HIV-1 strains identified in infected Cameroonians fail to react serologically in standard whole-virus ELISA, yet are readily detectable by conventional Western blot analysis (Simon et al., 1998).

Despite the heterologous nature of different HIV-1 isolates, and in particular, their envelope glycoproteins, a highly conserved and immunodominant domain, located within the gp41 envelope protein, still elicited antibody in infected individuals, which was reactive with HIV-1 samples of diverse geographic origin (Gnann et al., 1987).

2.3 HIV-1 Life cycle

Infection starts with absorption of virus particles through gp120 to CD4 molecules on the surface of susceptible cells. Their subsequent interaction with a coreceptor, one of which are known to be the seven membrane-spanning CC or CXC families of the chemokine receptors, is required for membrane fusion and entry (Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Feng et al., 1996). The two most important are CXCR4 and CCR5. Following entry, subviral particles are partially uncoated in the cytoplasm. The RNA is converted in the cytoplasm through the action of RT into double-stranded proviral DNA. The proviral DNA is transported to the nucleus and integrated into the host chromosome through the action of the viral integrase (Farnet and Haseltine, 1990).

The integrated viral DNA serves as a template for DNA-dependent RNA polymerase (pol II) and leads to the production of mRNAs that are translated into viral proteins in the cytoplasm of infected cells. Multispliced mRNA that encodes the nef, tat, and rev proteins is expressed early, and unspliced (encoding gag-pol polyproteins) and single spliced mRNA (encoding env, vif, vpu and vpr) is expressed late.

The translation of the mRNAs results in the gag-pol precursor polyprotein ($Pr160^{gag\text{-}pol}$) and the gag gene product polyprotein $pr55^{gag}$, among other proteins. The NC domain of the $pr55^{gag}$ polyprotein recognizes and binds to a sequence near the 5′ end of the progeny genomic RNA (Aldovini and Young, 1990; Sakaguchi et al., 1993). The $pr55^{gag}$ -RNA complex and $Pr160^{gag\text{-}pol}$ gather at the cell plasma membrane aided by the MA and NC domains of the $pr55^{gag}$. The MA domain of the $pr55^{gag}$ precursor is thought to interact with the cytoplasmic tail of the TM subunit (Dorfman et al., 1994; Lodge et al., 1994) of the vast number of env glycoprotein oligomers, which are inserted in the cell membrane (Bryant and Ratner, 1990; Gottlinger et al., 1989; Zhou and Resh, 1996).

During and after budding the viral protease in the $Pr160^{gag\text{-}pol}$ polyprotein cleaves the polyprotein, and a new virion is completed, containing a mature nucleocapsid composed of fully processed gag (MA, CA, NC, p6, p1 and p2), pol gene products (PR, RT, RNase H, and IN) and two molecules of genomic RNA.

2.4 Reverse transcriptase

2.4.1 Reverse transcription of genomic RNA

HIV-1 RT catalyzes the synthesis of proviral DNA, using viral RNA as a template. HIV-1 RT is a multifunctional enzyme that has three distinct activities: RNA-dependent and DNA-dependent DNA polymerase activity and RNase H activity that degrades the RNA–DNA intermediates formed during proviral synthesis.

The process of retroviral DNA synthesis is believed to follow the scheme outlined below:
1). The 3′ end of a partially unwound transfer RNA annealed to the primer-binding site (PBS) in the genomic RNA is used as a primer for minus-strand DNA synthesis. Minus-strand DNA synthesis proceeds until the 5′ end of genomic RNA is reached, generating a DNA intermediate of discrete length termed the minus-strand strong-stop DNA (–sssDNA).
2). Following RNase-H-mediated degradation of the RNA strand of the RNA: –sssDNA duplex, the first strand transfer causes –sssDNA to be annealed to the 3′ end of the viral genomic RNA, mediated by the repeated (R) sequences.
3). Minus-strand DNA synthesis resumes, accompanied by RNase H digestion of the template strand. This degradation is however, not complete.
4). A defined RNA segment derived from the short polypurine tract (PPT) primes plus-strand DNA synthesis. Plus-strand synthesis is halted after a portion of the primer tRNA is reverse-transcribed, yielding a DNA called plus-strand strong-stop DNA (+sssDNA).
5). RNase H removes the primer tRNA. This exposes the PBS at the 3′ end of +sssDNA, allowing the plus-strand DNA to transfer (second-strand transfer) and hybridize with the homologous region at the 3′ end of the minus-strand DNA.
6). Plus- and minus-strand syntheses are then completed, with the plus and minus strands of DNA each serving as template for the other strand.
A more detailed description of these steps can be found in reference papers (Gotte et al., 1999).

2.4.2 Molecular structure of HIV-1 RT

The HIV reverse transcriptase is a heterodimer consisting of 66 and 51 kd subunits. The 51-kd subunit (p51) is derived by proteolytic removal of the C-terminal 15-kd RNase domain of p66 by PR. The structure of HIV-1 RT has been determined by x-ray crystallography in a number of studies (Arnold et al., 1992; Esnouf et al., 1995; Huang et al., 1998; Jacobo-Molina et al., 1991; Jacobo-Molina et al., 1993; Kohlstaedt et al., 1992; Rodgers et al., 1995). The crystal structure of HIV-1 RT reveals a highly asymmetric folding in which the orientations of the p66 and p51 subunits differ substantially. The p66 subunit can be visualized as a right hand, with the polymerase active site within the palm, and a deep template-binding cleft formed by the palm, fingers and thumb subdomains (Kohlstaedt et al., 1992) (Fig.3).

The polymerase domain is linked to RNase H by the connection subdomain. The active site contains three critical Asp residues (Collins et al., 1998; Fornerod et al., 1997; Fouchier et al., 1997) in close proximity, and two coordinated Mg^{2+} ions. Mutation of these Asp residues abolishes RT polymerizing activity.

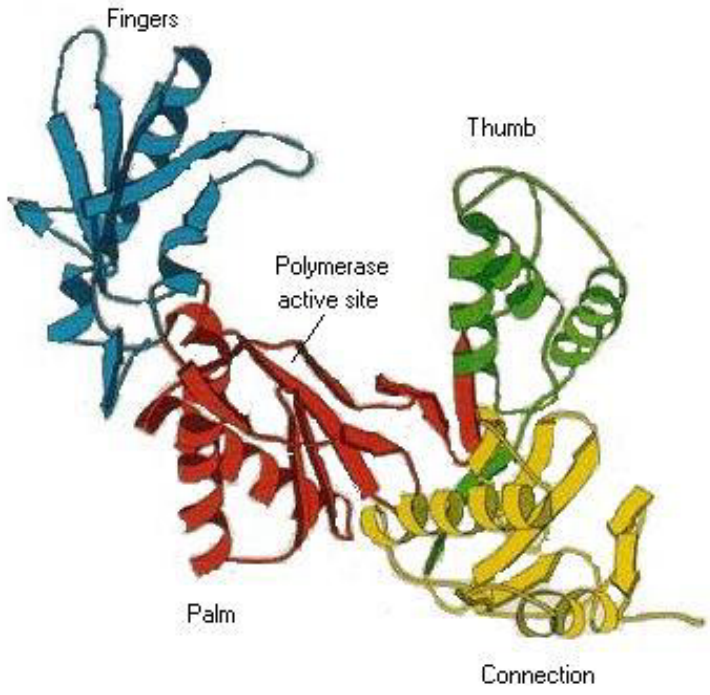

Figure 3. Ribbon drawings of the p66 subunit of HIV-1 RT (from Jacobo-Molina et al., 1993).

Approximately 18 base pairs of the primer-template duplex lie in the nucleic acid binding cleft, stretching from the polymerase active site to the RNase H domain. An RT-primer-template –dNTP structure model suggests that the fingers close in to trap the template and dNTP prior to nucleophilic attack of the 3´-OH of the primer on the incoming dNTP. After the addition of the incoming dNTP to the growing chain, it can be proposed that the fingers adopt a more open configuration, thereby releasing the pyrophosphate and enabling RT to bind the next dNTP (Huang et al., 1998). The RNase H activity acts in concert with the polymerizing activity, removing RNA in the DNA-RNA hybrid approximately 16-18 nucleotides from the polymerizing site.

The two RT subunits interact with each other in an asymmetric fashion. Only the large subunit contributes directly to the polymerase activity of the RT heterodimer (Hostomsky et al., 1992; Le Grice et al., 1991), there is no template binding cleft in p51. The p51 subunit of HIV-1 RT appears to have a structural role within the p66/p51 heterodimer; it may also be involved in binding the tRNA primer.

2.4.3 Biochemistry of reverse transcription

2.4.3.1 The DNA polymerase activity of RT

The general catalytic scheme of the DNA polymerase activity of RT involves first, binding of the primer-template by RT, then binding of the appropriate dNTP to the primer-template complex, followed by nucleophilic attack to yield the phosphodiester bond and the release of pyrophosphate. Continued synthesis requires RT to move relative to the template-primer. This movement must reposition the enzyme so that the additional nucleotides have access to the polymerase active site. Under normal processive DNA synthesis, the dissociation rate between RT and the template-primer becomes rate-limiting (Hsieh et al., 1993; Kati et al., 1992). RT can operate over relatively broad ranges of salt and pH and requires divalent cation Mg^{2+}. Although the natural template for RT is a heteropolymeric RNA, the enzyme is significantly more active on certain synthetic homopolymeric RNA templates. Homopolymer templates are commonly used to monitor RT activity, which has been used as a sensitive indication of the presence of retrovirus (Goff et al., 1981; Verma, 1977).

An important feature of RT is its lack of a proofreading function. RT does not contain a 3´exonuclease activity capable of excising mispaired nucleotides (Battula and Loeb, 1976) and is, as a result, more error-prone than cellular DNA polymerase which is capable of proofreading (Battula and Loeb, 1974). There is no 5´ exonuclease activity, so it cannot perform nick translation reactions. Strand displacement is, however, relatively efficient so that a nicked duplex DNA can serve as a template (Whiting and Champoux, 1994). The error –prone nature of RT leads to high mutation rates. Several studies (Boyer et al., 1992; Yu and Goodman, 1992) suggested that the misincorporation rate of most RTs in vitro, under physiological conditions, is on the order of 10^{-4} errors per base incorporated. This rate would translate into an error rate in viruses of about one per genome in a reverse transcription cycle, even without considering errors made by host RNA polymerase.

2.4.3.2 RT assays

The enzymatic activity of RT is routinely used in the laboratory to quantitatively monitor levels of virus present in the supernatant of infected cultures or to determine the effect of RT inhibitory substances.

The first generation RT assays were soluble assays based on the incorporation of a radioactively labeled nucleotide into an acid insoluble product (Baltimore, 1970). The RNA templates and complementing DNA primers used were usually synthetic homopolymers or alternatively heteropolymeric ribosomal RNA (rRNA) or endogenous RNA genomes of viral origin. The dNTPs used were usually labeled with ^{3}H or ^{32}P. The product was separated from unincorporated nucleotide by trichloroacetic acid (TCA) precipitation onto a glassfibre filter.

After several washes the radioactivity on the filter could be measured in a radioactivity counter. These assays suffered from several drawbacks such as low sensitivity and cumbersome handling with demands on specialized equipment for high throughput handling and measurement of samples.

Improved methods were developed utilizing substrates for colorimetric or chemoluminiscent product detection (digoxigenin-dUTP, biotin-dUTP, BrdUTP), thereby increasing detection sensitivity as an additional amplification step is introduced, and also offering less cumbersome processing of the samples (Eberle and Seibl, 1992).

To facilitate separation of substrate and product, as well as to enable sequential manipulation of the polymerization reaction, methods based on immobilized template or primer has been developed (Ekstrand et al., 1996; Gronowitz et al., 1991; Milavetz et al., 1977; Milavetz et al., 1978).

In addition, as RT is an immunogen, it can be measured non enzymatically using Western blot or ordinary immunoassays, i.e. specific HIV-1 RT ELISA. Detection down to approximately 10pg/ml of recombinant antigen has been demonstrated with the latter type of assay (Loveday and Tedder, 1993).

2.5 Course of HIV-1 infection

The course of HIV-1 infection can be divided into three stages: primary infection, clinical latency and clinically apparent disease. Primary infection with HIV-1 is often associated with an acute mononucleosis-like clinical syndrome that appears approximately 3–6 weeks following infection. Significant declines in the levels of CD4+ T lymphocytes in the peripheral blood occur in the first 2–8 weeks following HIV-1 infection (Gaines et al., 1990). These levels may rebound toward normal as the patient enters the clinically latent stage of disease (see below); however, they rarely if ever return to pre-infection levels. The acute syndrome associated with primary HIV-1 infection is accompanied by a burst of viral replication that can be detected in the blood approximately 3 weeks following infection (Fig.4)(Clark et al., 1991; Daar et al., 1991; Tindall and Cooper, 1991). During this period, infectious virus and viral proteins can be readily detected in the cell-free plasma as well as in cerebrospinal fluids, and the number of virions in cell-free plasma can reach 10^6 to 10^7 per milliliter. However, as for all retroviruses, only a small percentage of the virions are infectious.

Following the induction of an immune response to HIV-1 in humans, there is usually a relatively long period that is characterized by few, if any, clinical manifestations. When present, clinical symptoms are usually mild. Current estimates for the average time from infection with HIV-1 to the development of the clinical conditions that define AIDS vary from 8 to 12 years (Lemp et al., 1990b; Moss and Bacchetti, 1989). The estimated time has gradually increased since AIDS was first recognized. This may be due both to better detection of infection and to antiretroviral therapies and effective prophylaxis for life-threatening opportunistic infections (Lemp et al., 1992; Lemp et al., 1990a; Seage et al., 1993). Throughout the latent period, there is usually a steady decline in the numbers of CD4+ lymphocytes associated with continual viral replication. The level of virus is remarkably constant, increasing only gradually during the course of infection. Simple modelling suggests

that this relatively stable viral load reflects both a constant rate of new infection and death of the infected cells throughout most of the course of the infection (Coffin, 1995).

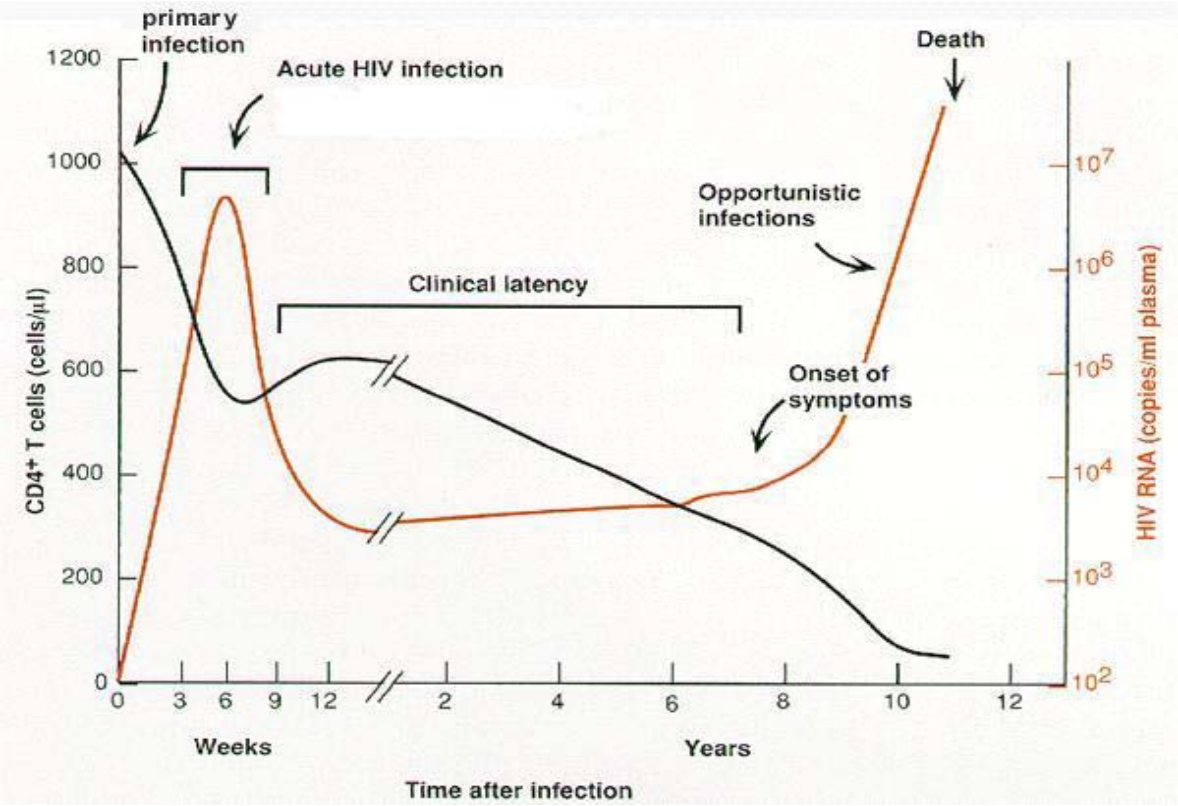

Figure 4. Typical course of HIV-1 infection (from Pantaleo et al., 1993).

After months to years of a continuous, yet variable, decline in the number of CD4+ T cells, the level of these cells drops from a normal range of 600–1200 to below 500 cells/µl which is associated with the conditions that define AIDS. At about 500 cells/µl, the first symptoms tend to appear in the HIV-infected patient. Once the CD4+ T-cell count falls below 200 cells/µl, the patient is susceptible to AIDS-defining opportunistic infections and neoplasms. The decline in the level of CD4+ T cells typically continues until virtually all such cells are lost.

2.6 Diagnosis and Laboratory monitoring

2.6.1 Diagnosis

The laboratory tests for diagnosis are based on 1) detection of host antibodies against the virus; 2) viral antigens; 3) viral nucleotide sequences, or 4) cultivation of the virus in cell cultures. The classic approach is to use a sensitive assay for detection of HIV IgG antibodies in serum as a first-line screening assay. A positive enzyme immunoassay test must always be followed by a confirmatory test. A Western blot assay is the most reliable confirmatory

serologic test for the diagnosis of HIV infection. A positive test demonstrates reactivity to at least two of the following HIV proteins: p24, gp41, gp120, and gp160. Indeterminate tests may be the result of reactivity to antigens that cross-react with HIV determinants; it also may be a reflection of recently acquired infection and an evolving antibody response. Therefore, clinical follow-up and repeated serologic testing is essential in these cases.

However, there are situations that require the use of additional assays. After infection, a period of two weeks to two months can be seen during which no antibodies are detected. In some cases when there is suspicion of an HIV infection and antibodies are not detected, especially with symptoms of primary infection, additional information may be required. Determination of the virus directly through an HIV-p24 assay or by using a molecular approach for the detection of viral RNA in serum can help to make the diagnosis.

2.6.2 Monitoring HIV-1 infection

2.6.2.1 Markers of HIV infection

The ideal laboratory marker of HIV disease activity should be easily and reproducibly measurable in all individuals with the infection. It should also worsen with progression of disease and improve with positive responses to therapy. CD4+ T cells are the primary targets of HIV infection and the depletion of CD4+ T cells is the immunologic hallmark of HIV disease progression. Measurement of CD4+ T cells is an excellent marker of disease progression. The plasma viral load is the most valuable prognosticator of HIV infection that is currently available. Viral load not only predicts disease progression independent of the CD4+ T-cell count, but also actually predicts the trajectory of the CD4+ T-cell count (Mellors et al., 1995b; Mellors et al., 1997).

2.6.2.2 Viral load monitoring

Before the development of currently commercialized sensitive molecular techniques for the quantitation of HIV RNA molecules, direct culture of HIV from plasma or mononuclear cells was found to correlate with stage of disease (Coombs et al., 1989; Ho et al., 1989). Changes in levels of culturable plasma viremia further serve as reliable indicators of activity of antiretroviral agents (Davey et al., 1993); however, these assays are extremely labor intensive and less sensitive than currently available molecular diagnostic tests.

Measurement of p24 antigenemia is relatively insensitive. Quantification of plasma p24 antigen encounters disturbing factors such as antigenic variation of p24, occurrence of anti p24 blocking antibodies and the presence of non-virion associated p24 (Corrigan et al., 1998; Jurriaans et al., 1995; Nadal et al., 1999). Although a positivity test for p24 antigenemia has prognostic value with regard to progression of disease, changes in levels of p24 antigenemia during antiretroviral therapy do not serve as an accurate surrogate for therapeutic efficacy during antiretroviral therapy (DeGruttola et al., 1994).

The ability to assess the level of viral replication with quantitative assays of HIV RNA in plasma (i.e., plasma viremia) has revolutionized the use of laboratory markers for HIV disease. Technology currently available for reproducible measurement of plasma viremia in clinical specimens includes PCR, nucleic acid sequence-based amplification (NASBA), and

branched DNA (b-DNA) assays. Each of these assays is able to detect as few as approximately 20 to 50 molecules of HIV RNA per milliliter of plasma, and each has a dynamic range of several orders of magnitude.

Plasma viral load serves as a valid marker not only for predicting disease progression but also for monitoring therapy in HIV-infected individuals. The development of sensitive, quantitative assays for plasma viremia, and the ability of this marker to predict progression of disease and to serve as an indicator of therapeutic efficacy has added an important dimension to decisions regarding initiation and maintenance of antiretroviral therapy in HIV-infected individuals.

Current recommendations regarding the initiation and maintenance of antiretroviral therapy rely heavily on the two best laboratory markers for HIV disease progression, the CD4+ T-cell count and the level of plasma viremia (Carpenter et al., 1998).

3. Anti-HIV-1 chemotherapy

A number of steps in the viral life cycle can be considered as potential targets for chemotherapeutic intervention: 1) viral adsorption, through binding to the viral gp120; 2) viral entry, through blockade of the viral coreceptors CXCR4 and CCR5; 3) virus–cell fusion, through binding to the viral gp41; 4) reverse transcription; 5) proviral DNA integration; 6) viral mRNA transcription; and 7) protease reaction.

Since the discovery of zidovudine (AZT) as an effective antiretroviral agent against HIV-1, drug therapy has been used widely in the treatment of AIDS. All the compounds that are currently used, or are subject of advanced clinical trials, for the treatment of HIV infections, belong to one of the following classes: (1) nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs); (2) non-nucleoside reverse transcriptase inhibitors (NNRTIs); and (3) protease inhibitors (PIs).

3.1 Mechanism of Anti-HIV-1 drugs

The NRTIs are chain terminators that block further extension of the proviral DNA during reverse transcription. NRTIs that have been approved by the FDA include six nucleoside analogues and one nucleotide analogue: AZT, didanosine (ddI), zalcitibine (ddC), stavudine (d4T), lamivudine (3TC), abacavir (ABC) and tenofovir disoproxil fumarate (TDF) (Fig. 5). TDF is the only FDA-approved nucleotide analogue. Both nucleoside and nucleotide analogues are prodrugs that must be phosphorylated by host cellular enzymes. Phosphorylated NRTIs compete with natural deoxynucleoside triphosphates (dNTPs) for incorporation into the newly synthesized DNA chains where they cause chain termination.

More than 30 structurally different classes of compounds have been identified as NNRTIs. They are specifically inhibitory to HIV-1 replication and target at a non-substrate binding site of the RT (De Clercq, 1998). Three NNRTIs have been licensed for clinical use in the treatment of HIV-1 infection: nevirapine (NLV), delavirdine (DLV) and efavirenz (EFV) (Fig. 5). Emivirine (MKC-442) (Nibbs et al., 1999) is in advanced (phase III) clinical trials, and others are in preclinical or early clinical development. The NNRTIs bind to a hydrophobic pocket (NNRTI-binding pocket) located between the ß6-ß10-ß9 and ß 2-ß13-

ß14 sheets of the p66 subunit (Hsiou et al., 2001). The NNRTI-binding pocket is close, but not contiguous with, the active site. The NNRTIs inhibit HIV-1 replication allosterically by displacing the catalytic aspartate residues relative to the polymerase binding site (Esnouf et al., 1995; Kohlstaedt et al., 1992; Spence et al., 1995).

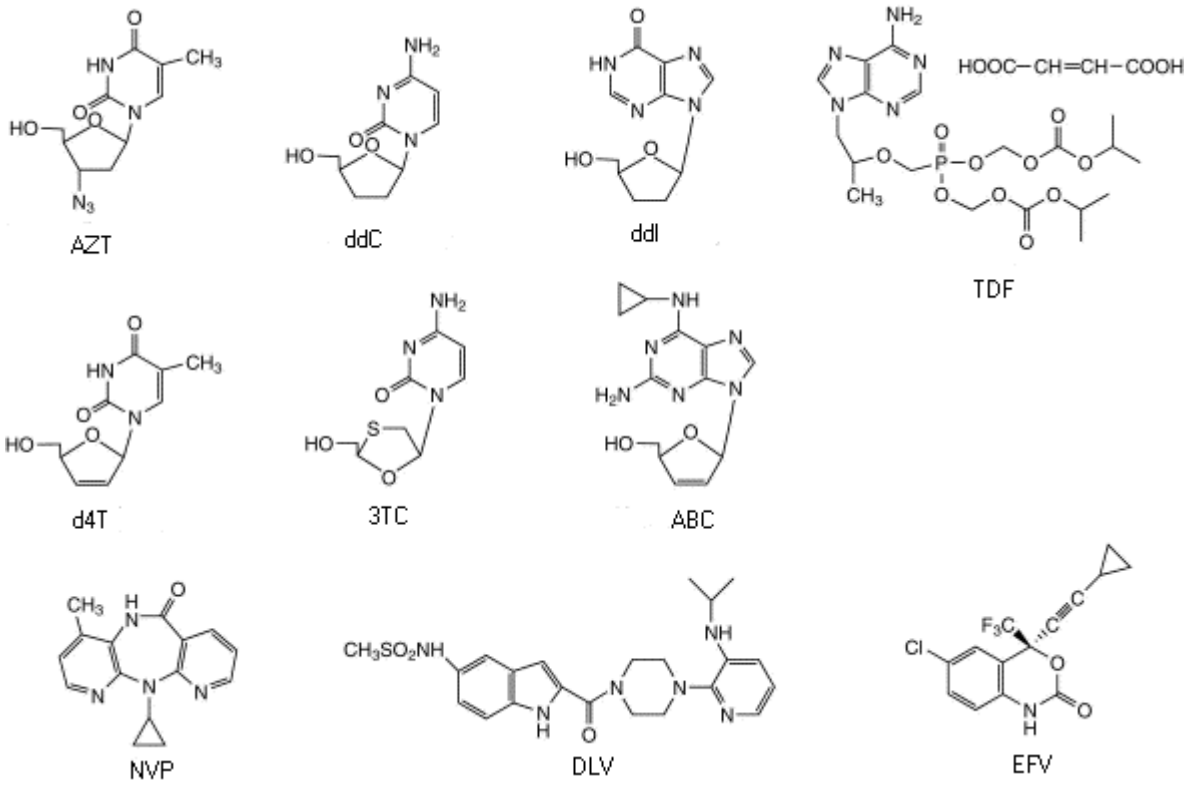

Figure 5. Chemical structure of NRTIs and NNRTIs that are licensed to treat HIV infection.

The HIV-1 protease enzyme is an aspartic protease composed of two non-covalently associated structurally identical monomers, 99 amino acids in length. Its active site resembles that of other aspartic proteases and contains the conserved triad, Asp-Thr-Gly at positions 25-27. The hydrophobic substrate cleft recognizes and cleaves 9 different sequences to produce the matrix, capsid, nucleocapsid, and p6 proteins from gag polyprotein and the protease, RT, and integrase from the gag-pol polyprotein. HIV PIs prevent the cleavage of the gag and gag-pol precursor polyproteins to the structural proteins and functional proteins (protease, RT/RNase H, integrase), thus arresting maturation and thereby blocking infectivity of the nascent virions (Flexner, 1998). All PIs that are currently licensed for the treatment of HIV infection, namely saquinavir, ritonavir, indinavir, nelfinavir, amprenavir and lopinavir, share the same structural determinant, i.e. an hydroxyethylene bond, which makes them nonscissile substrate analogues for the HIV protease. All six licensed PIs follow the same principle: they act as peptidomimetic inhibitors of HIV protease (Molla et al., 1998). In phase III clinical trials is atazanavir (BMS-232632) (Schols et al., 1997), which has been accredited with a favorable resistance profile that does not parallel any of the other PIs currently in clinical use, as well as a favorable pharmacokinetic profile that would allow once-daily dosing.

3.2 Combination therapy

AZT is the first drug approved for treatment of HIV infection (1987). Combination therapies that use two RTIs were introduced in 1994 (Eron et al., 1995; Hammer et al., 1996). The administration of at least two NRTIs and a protease inhibitor (PI) or NNRTI in combination resulted in sustained reductions of plasma HIV-1 RNA levels and, usually, larger increases of CD4+ T lymphocytes than had been seen with nucleoside analogue therapy (Gulick et al., 1998; Hammer et al., 1997). Combination therapy using any three or more drugs was termed highly active antiretroviral therapy (HAART). The widespread use of HAART was associated with an impressive decline in the morbidity and mortality of HIV-1 infected subjects (Law et al., 2000; Mocroft et al., 1998; Palella et al., 1998).

Advances in antiretroviral therapy since 1995 have made the concept of eradicating HIV a provocative possibility. However, antiretroviral failure is still common in the clinical setting. Drug failure could result from several factors, including intolerance, poor compliance, insufficient drug absorption, changes in drug metabolism and unfavorable drug interactions. Perhaps the most important factor, however, is drug resistance, particularly if the therapy was satisfactory for a long time before the viral load started to rise (Markowitz, 2000).

3.3 Drug resistance

The response to antiretroviral therapy in HIV infected patients is limited by the emergence of drug resistance. HIV drug resistance is mediated by mutations in the molecular targets of drug therapy. This resistance is a consequence of the high rate of HIV mutation, the high rate of viral replication (Coffin, 1995; Ho et al., 1995; Wei et al., 1995), and the selective effect of these drugs, which favors emergence of mutations that can establish clinical drug resistance.

3.3.1 Resistance to NRTIs

There are two biochemical mechanisms of NRTI drug resistance. The first mechanism is mediated by mutations that allow the RT enzyme to discriminate against NRTIs during synthesis, thereby preventing their addition to the growing DNA chain (Huang et al., 1998; Larder and Stammers, 1999; Sarafianos et al., 1999b). These substitutions of residues are close to the nucleotide-binding site of HIV-1 RT (Table 1). For example, K65R, T69D and L74V map to the β3-β4 loop in the fingers subdomain of p66, which folds over the triphosphate moiety of the incoming dNTP (Huang et al., 1998). The M184V substitution, which confers high-level resistance to 3TC, is close to the ribose ring of the incoming dNTP. The presence of a β-branched amino acid (valine, isoleucine or threonine) at position 184 interferes with inhibitor binding, partly because of a steric clash with the oxathiolane ring of the inhibitor (Sarafianos et al., 1999a).

Table 1. Amino acid substitutions in HIV-1 RT that are associated with antiretroviral drug resistance (modified from Menendez-Arias, 2002).

Antiretroviral drug	Amino acid substitutions
Nucleoside analogues	
AZT[a]	M41L, D67N, K70R, L210W, T215Y/F, K219Q/E
ddC	K65R, T69D, L74V, M184V
ddI	K65R, L74V, M184V
d4T	M41L, D67N, K70R, (V75T/M/S/A)[b], L210W, T215Y/F, K219Q/E
3TC	(E44D, V118I)[c], M184V/I
ABC	M41L, K65R, D67N, K70R, L74V, Y115F, M184V, L210W, T215Y/F, K219Q/E
Multiple nucleoside analogues	
151 complex	A62V, V75I, F77L, F116Y, Q151M[d]
69-70 insertion complex	M41L, A62V, insertion[d] (usually T69SSS, T69SSG or T69SSA), K70R, L210W, T215Y/F, K219Q/E
Acyclic nucleoside phosphonate	
TDF	K65R, L210W
NNRTIs	
Nevirapine	K103N, V106A, Y181C/I, Y188L/C/H, G190A/S
Delavirdine	L100I, K103N, Y181C/L, Y188L, G190E, M236L
Efavirenz	L100I, K103N, Y188L, G190S/A/T/Q
Multiple NNRTIs[e]	L100I, K103N, V106A, Y181C/I, Y188L, G190S/A, M230L

[a] In vivo, mutations that confer resistance to AZT might also be associated with cross-resistance to all NRTIs except 3TC.

[b] Observed in vitro but rarely found in patients after treatment failure.

[c] Together, these mutations confer low-level phenotypic resistance to 3TC in the absence of M184V/I.

[d] Amino acid substitutions that are crucial for the acquisition of the mutidrug-resistant phenotype, with exception for TDF.

[e] The presence of either K103N or Y188L alone or the combination of two or more of the other mutations substantially reduces the utility of all currently approved NNRTIs.

The second mechanism is mediated by nucleotide excision mutations (NEMs). Various combinations of these mutations at codons 41, 67, 70, 210, 215, and 219 (Harrigan et al., 1996; Hooker et al., 1996; Kellam et al., 1992; Larder and Kemp, 1989) have been shown to mediate ATP-dependent hydrolytic removal of a dideoxy nucleotide monophosphate (ddNMP) from a terminated cDNA chain (Arion et al., 1998; Lennerstrand et al., 2001a; Meyer et al., 1999; Meyer et al., 2000; Meyer et al., 1998) and to possibly cause a compensatory increase in RT processivity (Arts et al., 1998; Caliendo et al., 1996). RTs bearing these mutations showed no significant differences from the wild type enzyme in discrimination between AZT-TP and dTTP. During the past few years, several studies have shown that the NEMs are associated with clinical resistance not just to AZT, but also to d4T, ABC, and to a lesser extent, to ddI, ddC, and TDF (Miller et al., 2001; Wainberg and White, 2001). The structural basis underlying the NEMs mechanism of action is not yet fully understood. Modelling studies have suggested that the NEMs are in a position that would appear to increase the affinity of RT for ATP so that at physiologic ATP concentrations, excision is reasonably efficient (Boyer et al., 2001). The presence of the dNTP that would

have been incorporated next, in a ddNMP-terminated primer, had the primer been free for elongation, results in the formation of a stable "dead-end" catalytic complex between RT, primer, template, and dNTP (Boyer et al., 2001; Lennerstrand et al., 2001b; Meyer et al., 2000). The formation of such a dead-end complex interferes with the ability of NEMs to facilitate the resumption of virus DNA chain elongation. Modeling studies have suggested that the bulky azido group of AZT may interfere with the formation of a dead-end catalytic complex by sterically preventing the addition of the next dNTP.

M184V and several other NRTI-resistance mutations including L74V and possibly K65R (Larder and Stammers, 1999) interfere with the effect of the NEMs. The mutational antagonism between the NEMs and several of the mutations that act by allowing RT to discriminate against NRTIs probably explains the clinical synergism observed with certain dual NRTI combinations such as AZT/3TC, d4T/3TC, AZT/ddI, and d4T/ddI.

High-level resistance to both drugs in a dual NRTI combination usually requires multiple NRTI resistance mutations. Two genetic mechanisms of multidrug resistance have received much attention: (1) Q151M usually together with A62V, V75I, F77L, and F116Y; (2) a double amino acid insertion at position 69 in combination with T215Y/F and other NEMs. These two mutational patterns, however, are responsible for only a minority of multidrug resistant isolates. Multidrug resistance more commonly results from a combination of ≥4 NEMs, M184V, and 1 to 2 mutations in the ß2-ß3 loop, particularly at position 69.

3.3.2 Resistance to NNRTIs

The mutations responsible for NNRTI resistance are in the hydrophobic pocket which bind the inhibitors. They cluster in three major regions, which contain residues 98–108, 179–190 and 225–236 of the 66-kd subunit (Table 1). In addition, one residue (Glu138) of the 51-kd subunit is associated with resistance to NNRTIs. A single mutation e.g. K103N in this pocket may result in high-level resistance to one or more NNRTIs. Resistance usually emerges rapidly when NNRTIs are administered as monotherapy or in the presence of incomplete virus suppression, suggesting that resistance may be caused by the selection of a pre-existing population of mutant viruses within an individual (Conway et al., 2001; Havlir et al., 1996; Wei et al., 1995). Like many of the PI and NRTI resistance mutations, some of the NNRTI resistance mutations may also compromise virus replication. Two mechanisms of impaired replication have been proposed: changes in the conformation of the dNTP binding pocket (Kleim et al., 1994; Van Laethem et al., 2000) and changes in RNase H activity (Archer et al., 2000; Gerondelis et al., 1999).

The hydrophobic binding pocket to which the NNRTIs bind is less well conserved than the dNTP binding site. HIV-1 group O and HIV-2 (Descamps et al., 1997; Hizi et al., 1993; Shih et al., 1991; Yang et al., 1996) are intrinsically resistant to most NNRTIs.

3.3.3 Sensitization to antiviral drugs by drug-specific resistance mutations

The development of new inhibitors of RT and PR might make it possible to administer combinations of drugs that have synergistic effects on viral suppression to improve drug

combination therapies. When incorporated into wild type RT, several mutations sensitize the enzyme to RT inhibitors (Balzarini, 1999). For example, the substitution Q161L, which appears during foscarnet (PFA) treatment, confers a 11-fold increase in susceptibility to AZT (Mellors et al., 1995a). By contrast, there are several mutations that reverse the phenotypic resistance of HIV-1 RT variants with drug-resistance backgrounds, such as L74V, M184V, L100I and Y181C (Gu et al., 1995; Larder, 1992; St Clair et al., 1991; Tachedjian et al., 1996).

Although the M184V mutation does not affect the incorporation of AZT-TP, either in the presence or in the absence of AZT resistance related mutations, it alters the active site of RT polymerase in a way that compromises the rescue of ATP-mediated excision of AZT-MP from the end of the DNA primer strand (Gotte et al., 2000). Despite the suppressive effect of M184V and other mutations that mediate AZT re-sensitization, viruses with dual resistance to the corresponding pair of drugs have been identified.

3.4 Drug resistance testing

Resistance of HIV-1 to RT or protease inhibitors can be determined genotypically by examining resistance-related mutations in the viral genome, and/or phenotypically by measuring the inhibition of virus replication or enzymatic activity of RT/protease by specific drugs. To date, several phenotypic and genotypic assays have been developed and are being used to characterize drug resistance.

3.4.1 Phenotypic assays

A standardized assay is available for determination of drug susceptibilities of most HIV-1 isolates in peripheral blood mononuclear cells (PBMC) (Japour et al., 1993). The method involves cocultivating patient PBMC with seronegative donor PBMC to generate a virus stock; titrating the virus stock to determine the virus infectivity, using a standardized inoculum (on the basis of the infectivity properties of the stock) to infect cultures at varying concentrations of an antiretroviral agent; and then calculating IC_{50} on the basis of a measure of infection (HIV-1 p24 antigen levels). This assay has several limitations. It is labor intensive and time consuming, taking a minimum of 6 weeks from the time of initial specimen collection to generation of data. Furthermore, the assay may impose significant in vitro selective pressures that may favor the outgrowth of certain virus isolates and not others from the original heterogeneous virus population. This method determines the susceptibility of virus present in a patient's infected PBMC and does not assess plasma virus directly.

To circumvent some of the above problems, several recombinant virus assays (RVA) have been developed (Hertogs et al., 1998; Kellam and Larder, 1994; Martinez-Picado et al., 1999; Petropoulos et al., 2000; Shi and Mellors, 1997). These assays are based on direct amplification of the patient's virus–derived gene sequence of interest (protease, reverse transcriptase [RT], or both) from viral RNA in plasma, by means of RT–PCR. The PCR product is inserted into a modified HIV-1 vector that lacks the analogous sequence. A recombinant virus is produced and then used in rapid drug susceptibility studies.

To date, two RVAs are commercially available to measure susceptibility to RT and protease inhibitors: Virco Antivirogram and ViroLogic PhenoSense. Both assays amplify HIV-1 RT

and protease but differ in technical aspects related to recombinant virus construction and detection of virus replication. Drug susceptibility results are reported as IC_{50} values for each drug and fold differences of these values relative to a reference wild type virus. Fold differences >4 and >2.5 are considered evidence of decreased susceptibility for the Antivirogram and PhenoSense assays, respectively. The Antivirogram assay requires plasma with ≥1000 HIV-1 RNA copies/ml, while the PhenoSense assay can be done on plasma with ≥500 HIV-1 RNA copies/mL, Both tests are generally completed in 8–10 days, and the practical turnaround time to clinicians, including shipping, is 2–4 weeks.

The above replication-based assays depend on multiple unstandardized factors including the inoculum of virus tested, the cells used for virus replication, and the means of assessing virus replication. Susceptibility testing of NRTIs is further complicated by the fact that NRTIs are triphosphorylated to their active form at different rates in different cell lines

An Amp-RT based assay has also been described for the analysis of RT susceptibility to NRTIs and NNRTIs. It detects RT activity by using a known heterologous RNA template. The RT-derived cDNA is amplified by PCR and detected by an ELISA-based hybridization with an probe (Garcia Lerma et al., 1998; Heneine et al., 1995; Yamamoto et al., 1996). Phenotypic resistance to RT inhibitors is measured by quantitating the RT signal generated in Amp-RT reactions done in the presence and absence of the relevant RT inhibitor. RT activity may be disturbed by environmental factors, e.g. cellular polymerase, RT blocking antibodies and antiviral drugs in this assay.

A limitation for current phenotypic resistance testing is the lack of consensus on what increase in the IC_{50} of a drug is clinically significant. This increase is likely to be different for different antiretroviral agents and is related to the blood levels of the drug.

3.4.2 Genotypic assays

All genotypic assays currently used are based on PCR amplification of viral *pol* sequences primarily from plasma by RT-PCR. Amplified products can be analyzed by hybridization or sequencing techniques.

Dideoxynucleotide sequencing has become a standard method. It relies on automated sequencers. Sequence alignment and editing, followed by mutation detection and interpretation, have been increasingly automated with recently introduced HIV-1–specific kits and software (TrueGene HIV-1 genotyping kit and OpenGene DNA sequencing system [Visible Genetics]; ViroSeq HIV-1 genotyping system [PE Biosystems]). This automation has reduced the time from receipt of a specimen to final report of data to as few as 2 days, and practical turnaround time to clinicians ranges from 3 to 21 days.

Hybridization methods determine the sequence based on defined oligonucleotide probes. The entire protease and most of the RT sequence can be determined through sequencing by hybridization to miniaturized high-density arrays of oligonucleotide probes, followed by automated nucleotide base calling and mutation detection (GeneChip; Affymetrix) (Kozal et al., 1996). More limited sequence information can be obtained by use of a hybridization-based line probe assay (LiPA; InnoGenetics), which rapidly and simultaneously determines the presence of pre-selected drug resistance mutations (Stuyver et al., 1997).

Although genotyping assays are generally faster and less expensive than phenotypic assays, they have a major limitation in relation to the interpretation of their results. The contribution of some mutations to phenotypic resistance is controversial. Even well-described resistance mutations can interact in complex ways to alter phenotypic susceptibility and cross-resistance to other drugs (Hanna and D'Aquila, 1999).

There are several common limitations for all current drug resistance tests. For example, (1) The HIV-1 population within an individual consists of innumerable variants. Detection of clinically significant minority species remains challenging. (2) Problems with specimen mix-up, PCR contamination, and technical performance may compromise the quality of data from some clinical laboratories. A recent study that used well-defined coded samples has shown large inter laboratory differences in the quality of the results obtained by automated DNA sequencing in 23 laboratories worldwide (Schuurman et al., 1999). These findings highlight the need for proper quality control and standardization among currently used sequencing methods.

Several retrospective clinical trials have suggested that resistance testing may be useful in the assessment of the success of salvage antiretroviral therapy. Prospective, controlled trials have demonstrated that resistance testing improves short-term virological response. Resistance testing is currently recommended to help guide the choice of new drugs for patients after treatment has failed and for pregnant women. Resistance testing should also be considered for treatment-naïve patients, to detect transmission of resistant virus.

4. AIMS OF THE STUDY

To develop a set of sensitive non-radioactive RT activity assays that can be used for:

1. Screening of potential HIV-1 RT inhibitory substances and analysis of their mode of action (I, II).

2. Characterization of RT from AZT resistant HIV-1 isolates from cell culture (III).

3. Monitoring HIV-1 viral load by quantification of RT activity recovered directly from plasma (IV).

4. Phenotypic drug susceptibility testing of HIV-1 based on plasma derived RT activity (V).

5. RESULTS AND DISCUSSION

5.1 RT assays developed for this study

RT assay can be used for demonstration of the presence of retrovirus, for enzymatic studies of reverse transcriptase and for screening and characterization of RT inhibitors. The first generation RT activity assays were based on measuring the incorporation of dNMP into DNA, using a radioactive dNTP as substrate (Baltimore, 1970). All reaction components were mixed together in solution. The enzyme activity was determined as amount of acid precipitable product. These methods were insensitive and labor intensive.

A non- radioactive RT assay has been developed by our group (Ekstrand et al., 1996). The assay is based on immobilized template in 96-well microtiter plates, and uses non-radioactive BrdUTP as substrate. The incorporated BrdU is detected immunologically by alkaline phosphatase (AP) conjugated anti-BrdU antibody, which is quantified colorimetrically by addition of the AP substrate solution containing p-nitrophenyl phosphate (pNPP). The sensitivity of the assay can be varied by altering the RT reaction duration and/or by prolonging the AP reaction. The assay can detect <0.02 pg of recombinant HIV-1 RT (HIV-1 rRT) per well. This assay has recently been modified with a higher template-primer amount and fluorescent product detection by addition of the AP substrate 4-methylumbelliferyl phosphate (MUP). The sensitivity has been increased up to 0.25 fg RT derived from HIV-1 infected plasma in each well, when using overnight RT reaction duration and 2 h AP reaction time.

The RT reaction sequence consists of several steps (see 2.4.3.1). An RT inhibitor may act on any of these steps. The solid phase coupled template can be used as an affinity matrix for the binding of RT, it allows for sequential reaction steps separated by washes without the loss of template and product. We therefore developed a number of different RT assays for characterization of HIV-1 RT from various sources, for screening of potential RT inhibitory substances, and for analyses of the mode of action of RT inhibitors.

5.1.1 Standard RT inhibition assay (IC assay)

The standard RT inhibition assay is used for determination of IC_{50} values of RT inhibitors. The procedure is outlined in Table 2 and illustrated in Figure 1(a), Paper I. The IC_{50} value is the drug concentration giving 50% inhibition of RT activity.

Different reaction solutions can be used for different purposes. BrdUTP and odT primer were used at concentration close to their Km values in titration of NRTIs. For NNRTIs that are not dependent on BrdUTP and primer concentration saturated concentration were applied (Paper I, II, III, V).

Since the concentration of BrdUTP or primer can be varied in the reaction solution, it is possible to determine if an inhibitor is competing with either the natural dNTP substrate or the primer in the IC assay. The IC_{50} values should be dependent on BrdUTP concentration for

an inhibitor competing with substrate, and dependent on primer amount for inhibitors that compete with primer.

Table 2. A schematic illustration of the steps in different assays for analyses of RT-inhibitors.

Step	IC assay	BIC assay	BEC assay	CT assay
1		Add inhibitor to binding buffer containing RT, with or without primer	Add inhibitor to binding buffer containing RT, with or without primer on an RT bound plate	Add inhibitor to reaction solution containing primer, with or without RT, without BrdUTP
2		Incubation for 90 min at 33°C	Incubation for 90 min at 33°C	Incubation for 4 h or overnight at 33°C
3		Wash	Wash	Wash
4	RT reaction step. Add inhibitors to reaction solution containing BrdUTP, primer and RT.	RT reaction step. Add reaction mixture including BrdUTP, primer if no primer was added in step 1.	Add Mouse anti-RT MAb	RT reaction step. Add reaction mixture including BrdUTP, with or without extra RT.
5	Incubation for 3 h at 33°C	Incubation for 3 h at 33°C	Incubation for 90 min at 33°C	Incubation for 3 h at 33°C
6	Wash away unbound BrdUTP	Wash away unbound BrdUTP	Wash away unbound mouse MAb	Wash away unbound BrdUTP
7	Add anti-BrdU MAb conjugated with AP	Add anti-BrdU MAb conjugated with AP	Add goat anti-mouse Ab conjugated with AP	Add anti-BrdU MAb conjugated with AP
8	Wash away unbound Ab	Wash away unbound Ab	Wash away unbound Ab	Wash away unbound Ab
9	Detect the product with AP substrate	Detect the product with AP substrate	Detect the product with AP substrate	Detect the product with AP substrate
10	Read the plate	Read the plate	Read the plate	Read the plate

Note: All the assays were processed in a 96-well microtitre plate with poly(rA) template bound to the bottom of the wells.

The reaction buffer can also be modified according to the mechanism of action of the RT inhibitors analyzed. For example, in the buffer for characterization of AZT resistant viruses ATP or GTP was supplemented in order to support an energy dependent dideoxynucleotide excision reaction (Paper III, V).

5.1.2 RT binding inhibition assay (BIC assay) (I, II)

The BIC assay was designed to investigate if the inhibition found in the IC assay is due to prevention of RT binding to template or template-primer (Table 2). RT is first allowed to bind to template or template-primer in a binding buffer in the presence of dilutions of the RT inhibitors or a control. The binding step is terminated by a wash of the plate. The RT reaction solution is then added and the amount of bound RT activity is determined.

The inhibition found may be due to two reasons: 1) The inhibitor prevents RT binding to template or template-primer complex; 2) The inhibitor binds directly to the RT and thereby inactivates the activity. The amount of bound RT protein can be determined as below.

5.1.3 ELISA for RT protein detection (BEC assay)(II)

An ELISA was designed for detection of RT protein (Table 2). The RT is first incubated with template or template-primer in the presence of dilutions of an RT inhibitor or a control as in the BIC assay. The binding is terminated by a wash of the plate. The anti-RT MAb hybridoma supernatant and goat anti-mouse polyclonal antibody conjugated with AP are added to the plate sequentially with a wash after each step. The amount of bound RT protein is then quantified by addition of pNPP to the plate.

This assay specifically quantifies the amount of bound RT protein in a polymerase activity independent manner. An inhibitor that shows inhibitory effect in both the BIC assay and the BEC assay interferes with the binding of RT to template-primer.

5.1.4 Template-primer destruction assay (TDC assay) or chain termination assay (CT assay) (I, III)

The immobilized template makes it also possible to study capacity of inhibitors to inactivate template or primer. Such an assay was named TDC assay in Paper I and CT assay in Paper III. As outlined in Table 2, RT is present or absent in the first incubation with different dilutions of the RT inhibitors in a reaction solution devoid of BrdUTP (this step is named the destruction step). After a wash of the plate, a second reaction solution devoid of primer but including BrdUTP and additional RT is added. The function of residual template-primer is analyzed according to the standard procedure.

Chain terminators such as AZT-TP give RT inhibition only in presence of RT during the template-primer destruction step, and lower inhibition concentrations are required than in the IC assay, as no competitive BrdUTP is present. Any inhibition found independent of the presence of RT during the destruction step, on the other hand, is due to direct interaction of the inhibitor with template or primer.

The primer amount is a critical factor in TDC/CT assay when analyzing nucleotides that incorporate into the newly synthesized DNA chain and cause chain termination. The higher the primer amount used, the less the chain termination can be observed when a limited RT amount is used. The relationship between CT_{50} values and the primer amount was determined for AZT-TP (Fig. 1A, Paper III). A decrease in primer amount resulted in decreasing primer

function until the primer molecules reduced to a very low level, when RT amount exceeded that of primer.

5.2 Use of RT assays for dissection of the mode of action of RT inhibitors and screening of RT inhibitory substances with recombinant HIV-1 RTs (I, II)

To document the capacity of the presented assays for resolution of drug mechanism of action a set of reference substances were selected and analyzed to exemplify the typical results for different types of inhibitors. The results were summarized in Tables 2-4 in Paper I.

The IC_{50} value for nucleotides, such as dTTP, that can be incorporated into product showed a dependence on substrate but not primer concentration. Chain terminating nucleotides, such as ddTTP and AZT-TP, gave a similar pattern as dTTP, but displayed lower IC_{50} values. The CT_{50} values for ddTTP and AZT-TP were more than 50-fold lower than their IC_{50} values, while dTTP did not give a CT_{50} value.

PFA is a non-competitive inhibitor thought to interfere with the release of pyrophosphate (reviewed by Sandström and Öberg, 1993). Its IC_{50} value showed a dependence on both BrdUTP and primer, the value decreased with increasing primer amount. No inhibition was found in the BIC and the CT assays.

$P(dA)_{12-18}$ and prI, which can hybridize to the primer, exhibit both IC_{50} and BIC_{50} values that were dependent of primer but independent of substrate concentration. They destroyed the template-primer whether or not RT was present during the destruction step of CT assay. In contrast, the inhibition of poly(U), which can hybridize to the template, was independent of primer and dNTP substrate. Similar 50% inhibitory values were obtained with all three assays for poly(U) no matter if primer was present in the binding step of the BIC assay or if RT was present in the destruction step of the CT assay.

Poly(rG), which cannot hybridize to either primer or template, still displayed an IC_{50} value that is considerably higher than for $p(dA)_{12-18}$ and poly(U). Its BIC_{50} value was much lower than the corresponding IC_{50} and TDC_{50} values. This is probably because it can bind to RT and prevent RT binding to template or template-primer.

To summarize, different types of inhibitors exhibited different behaviors in relation to their mechanism of action. The combined use of the presented assays provides a way to dissect the mechanism of action of RT inhibitors.

NNRTIs 9-Cl-TIBO, NVP, DLV and MSA-300 (N-[*cis*-2-(2-hydroxy-3-acetyl-6-methoxyphenyl)-cyclopropyl]-N'-(5-chloropyrid-2-yl)-thiourea) were further analyzed for their mechanism of RT inhibition. All four NNRTIs tested showed inhibitory effects in the IC assay (Table 1, Paper II). However, no significant inhibition was found for 9-Cl-TIBO and NVP in BIC assay, which indicated that they did not inhibit RT binding to template or template-primer and that their binding to the RT protein was reversible.

MSA-300 gave the most prominent inhibitory effect of all investigated substances in both IC and BIC assay. This inhibition was not due to prevention of RT binding to template-primer

according to the results from BEC assay. It should thus be due to the inhibition of the catalytic capacity of the bound enzyme. The fact that MSA-300 displayed a BIC_{50} value in the same range as its IC_{50} value suggests that this inhibitor either binds to the enzyme irreversibly or has a much higher affinity for RT template-primer complex than the other inhibitors. This is an interesting observation that deserves further study for the interaction of MSA-300 and RT.

The phenomenon that BIC_{50} values for MSA-300 and DLV were higher in the absence of primer than in its presence during binding step suggests that their binding affinity to RT template-primer ternary complex is higher than to RT template binary complex.

The mechanism of action of NNRTIs revealed is in accordance with findings that they are, in general, non-competitive inhibitors with respect to template-primer, and their binding to RT leads to a conformational change which affects the active site (Ding et al., 1995; Kohlstaedt et al., 1992; Ren et al., 1995) (see also 3.1).

When comparing the current systems with the soluble RT assays or a radioactive RT assay based on carrier bound template primer (Gronowitz et al., 1992), similar IC_{50} values were obtained for most reference nucleoside substances when the same type of template-primer was used (Fan et al., 1995).

5.3 Characterization of RT from AZT-resistant HIV isolates from cell culture (III)

AZT monotherapy commonly leads to the development of resistant virus. This resistance is found to be associated with mutations in the RT gene, leading to defined amino acid (aa) substitutions at codons such as 41, 67, 70, 210, 215 and 219 (Kellam et al., 1992; Larder, 1992; Larder et al., 1989; Larder et al., 1991; Larder and Kemp, 1989). The corresponding mutant virus has been shown to be up to several hundred times less sensitive to AZT inhibition in cell culture than wild type isolates. However, only minor or no differences were revealed when comparing the kinetic properties of mutant enzymes with wild type (Carroll et al., 1994; Eron et al., 1993; Lacey et al., 1992).

During the last few years AZT resistance associated mutations were found to enhance the removal of AZT-MP from terminated cDNA chain by nucleotide excision mutations (see 3.3.1) (Arion et al., 1998; Lennerstrand et al., 2001a; Meyer et al., 1999; Meyer et al., 2000; Meyer et al., 1998). An increase in AZT-TP resistance of up to five times was demonstrated with RT having mutations D67N, K70R, T215F, and K219Q (Arion et al., 1998; Meyer et al., 1999), and a 10-fold increase in IC_{50} values of HIV RT was observed in parallel with our study (Lennerstrand et al., 2001a). However, this difference is still much smaller than that found in cell culture.

The IC assay and the CT assay were modified to characterize the enzymes from 18 HIV-1 isolates with various sensitivities to AZT in cell culture. The aim was to evaluate if enzyme assays could predict the susceptibility of virus to AZT, especially if CT assay could mirror the scale of difference found in cell culture between AZT sensitive and resistant viruses.

5.3.1 In the IC assay

In order to mimic the physiological conditions 0.5 mM GTP was included in the reaction mixture to support a nucleotide excision reaction. The total variation in sensitivity to AZT-TP inhibition among RTs investigated in IC_{50} assay was approximately 9-fold (Table 2). This was a significantly larger span compared to what was achieved earlier using a reaction mixture devoid of ribonuclotides (Lennerstrand et al., 1996).

A weak correlation was found between the IC_{50} value of RT and the ED_{50} value determined in cell culture ($r = 0.60$, $p<0.01$, Fig. 4, Paper III). However, no correlation was observed between the effect of AZT on HIV-1 multiplication in cell culture and the presence of AZT resistance associated aa substitutions (Table 2, Paper III). This was unexpected but might be due to the fact that the HIV-1 isolates studied in ED_{50} analysis were not from the same passages as used for the IC assay, the CT assay and sequence analysis. In addition, ED_{50} analysis for the tested isolates was done during a five-year period. Some modifications of the method have been made during this period, and between assays variation may be significant. A panel of site directed mutants originating from the same wild type virus would be a better material for this type of study.
The mentioned IC assay was later further improved for analysis of drug susceptibility using RT derived from plasma (see 5.4.2).

5.3.2 In the CT assay

Of the HIV-1 samples analyzed, isolates numbered 80, 157, 134 and 143 exhibited the highest CT_{50} values, and also higher CT_{50} : IC_{50} ratios than the other isolates studied. The span between the smallest and largest values was more than 600-fold (Table 2, Paper III).

The results from the CT assay revealed a tendency to obtain higher CT_{50} values when the number of AZT specific substitutions increased. However, two isolates deviated from this pattern. Isolate 80, which exhibited the highest CT_{50} value, contained only one AZT resistance associated mutation K70R in RT. Isolate 157, giving a CT_{50} value of 100 nM, had only substitutions M41L and L210W. Most surprisingly, both these RTs were found to contain the substitution T39A, which was not present in the RT of any other isolate (Table 2, Paper III). This suggests that RTs containing T39A substitution require significantly higher AZT-TP concentrations for primer termination in CT assay than other isolates.

Isolates 174 and 157 are isogenic in the context of the mutations tabulated but for the presence of T39A in isolate 157. These two isolates have large differences in CT_{50} values but identical IC_{50} value (Table 1, Paper III). The effects of T39A mutation in CT assay seem to be eliminated in presence of BrdUTP. This is in accordance with the observations of Meyer et al 1999, that the removal of ddNMP from the primer end by mutant HIV RT was efficiently inhibited by the dNTP complementary to the next base in the template, so called dead end complex (DEC) formation (see 3.3.1).

T39A is not a common polymorphism. It was originally described as a mutation occurring in an isolate that was co-resistant to AZT and PFA (Tachedjian et al., 1998). Recent data indicated that this substitution occurred in connection with NRTI therapy. It would be interesting to verify the function of T39A by studying site directed mutants.

The higher fold increase observed in the CT assay, compared to the IC assay, for RTs containing NEMs can be explained with two additive reasons: the lack of competition for BrdUTP incorporating into the newly synthesized DNA strand in the CT assay, and DEC formation in the IC assay.

As a summary of Paper III, the modified RT inhibition assay showed some capacity to predict the susceptibility of the virus to AZT in cell culture, and the CT assay revealed a more than 600-fold difference in primer termination between the different isolate RTs. These differences were related to the occurrence of certain mutations in the RT gene.

5.4 Characterization of HIV-1 RT in human plasma (IV, V)

Direct quantification and characterization of HIV RT in plasma is usually hampered by the disturbing factors such as cellular polymerase, RT blocking antibodies and presence of anti-HIV drugs. Having access to a panel of sensitive colorimetric and fluorimetric RT assays we aimed to develop procedures for separation of HIV RTs from such disturbing factors. The purified RTs can then be used for monitoring viral load and analysis of drug susceptibility.

5.4.1 HIV-1 viral load determination based on RT activity recovered from human plasma (IV)

The separation procedures for purification of RT/virion from plasma consists of three steps: 1) treatment of the plasma to inactivate cellular enzymes; 2) immobilization of the virions on a gel to allow a wash for removing disturbing factors including RT blocking antibodies; 3) lysis of the immobilized virions and elution of the viral RT. The RT activity recovered is then quantified employing RT assays with either a colorimetric or a more sensitive fluorescent detection of the DNA product.

5.4.1.1 Important factors for purification of HIV-1 RT from plasma

There are four major concerns for purification of RT on a gel: (1) the capacity of the gel for virion binding; (2) removal of cellular enzyme activity; (3) removal of RT blocking antibodies; (4) removal of the effect of antiviral drugs in plasma.

From a panel of 82 different chromatography media an ion exchange medium, Fractogel EMD TMAE Hicap, was chosen as binding gel based on its virion binding capacity and physical properties such as stability and particle size.

High titers of RT blocking Ab were one of the most prominent obstacles to overcome. Using optimized separation conditions the immunoglobulin (Ig) amount recovered was reduced more than 1000-fold (Fig.1B, Paper IV).

Examination of the unprocessed plasma samples revealed presence of large amounts of polymerase activity with capacity to utilize poly(rA) as template. The separation procedure reduced this activity approximately a 100- to 1000-fold but did not totally exclude it from detection (Table II, Paper IV).

The plasma treatment step was thus introduced to achieve reliable quantification of plasma containing small amounts of HIV particles. The principal behind this idea is that the treatment inactivates enzymes that are not protected by a condensed viral particle. Sulfhydryl reactive agents, e.g. N-ethyl malemeid have been widely used for specific elimination of the activities of cysteine dependent polymerases (Hubscher et al., 2000). Furthermore, the HIV-1 RT is extremely resistant to sulfhydryl reactive agents (Fig. 3, Paper IV). The consensus sequence of HIV-1 contains only two cysteine residues and HIV-2 three. Chemical modification of these cysteines is either not possible at normal conditions or does not affect the RT activity of the enzyme (Hizi et al., 1992). Recombinant RT containing the Y181C substitution was in contrast with the parental wild type RT found to be quite sensitive to cysteine modifying agents but not inhibited by the trace amounts recovered in lysate.

To control if antiviral drugs from plasma are recovered in the lysate and might disturb viral load measurement, human blood donor plasma spiked with at least two times maximum blood drug concentration were analyzed. The inhibitory effect of EFV and DLV that remained in 75 μl of lysate could be detected (Fig. 6). No inhibition was found for the other drugs. There was approximately 40% of inhibition by EFV recovered in lysate from the plasma with maximum EFV concentration that patients could have, when 15 μl of lysate was used in the assay.

5.4.1.2 Measurement of the RT load in human plasma

To demonstrate the usefulness of the RT purification method, plasma from healthy blood donors and coded plasma from HIV infected individuals were analyzed for RT load with both colorimetric and fluorescent DNA detection.

198/202 of blood donor samples were negative and four gave weak signals. The specificity of the assay was >98%.

The RT load results in relation to genome copies present in plasma are summarized in Table V, Paper IV. The correlation between plasma RT activity and plasma HIV RNA copies was highly significant ($R=0.90$, $p<0.0001$, Fig. 4, Paper IV). All samples holding >6900 RNA copies/ml were RT positive, while 50 % of the samples having 2500-6900 copies/ml and 10 % with 50-2500 copies/ml were also positive. Only one of 143 genome negative samples showed detectable RT activity.

The HIV infected individuals in the cohort can be classified into three groups according to treatment status: naïve, on pause, and on therapy. The three groups generated similar regression lines for the correlation between RT load measured and RNA copies (Fig. 4, Paper IV). This indicated that the drugs in patient plasma (including EFV) did not significantly alter the recovery of RT activity in the RT load assay, which is supported by our longitudinal data (Table VI, Paper IV)) and data in a parallel study (Braun et al., 2003).

PCR has problems in quantifying some HIV-1 subtypes. A study on a panel of 11-coded plasma from NIBSC, spiked with 10 different HIV-1 subtypes including group O and N and one negative control, showed that the RT load assay could quantify RT from all the different HIV subtypes tested. This is a remarkable advantage of the RT load assay compared to nucleic acid amplification techniques.

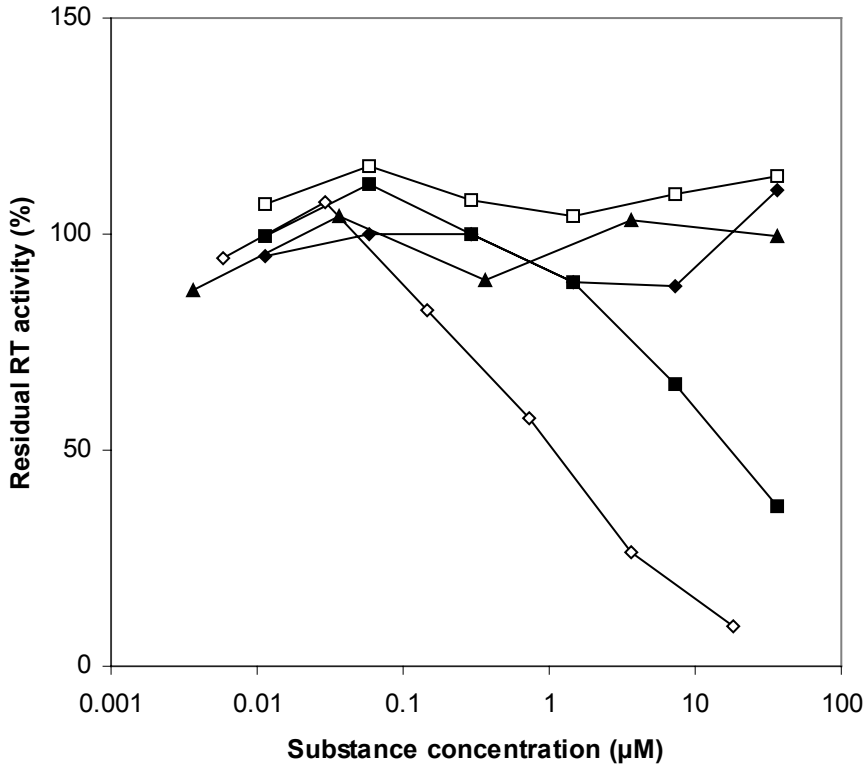

Fig. 6 Effect of antiviral drugs recovered in lysate on wild type HIV-1 RT. The drugs tested were: nevirapine (◆), efavirenz (◇), delavirdine (■), AZT-TP (□) and d4T-TP (▲). The concentrations of drugs in spiked human blood donor plasma were at least two times maximum blood drug concentration. 75 µl lysate was used in the RT assay.

5.4.1.3 Sensitivity of the RT load assay

The sensitivity of an RT assay is critical for measurement of viral load. Using fluorescent detection of DNA product the current cohort of HIV-1 plasma samples gave a detection limit of 5.4 fg RT/ml plasma on average, corresponding to 900 RNA copies/ml. When blood donor plasma spiked with HIV infected individual plasma was analyzed for sensitivity, the detection limit corresponded to an RT activity of 2.0 fg RT/ml or 374 RNA copies/ml plasma.

The discrepancy of detection limit in two sets of samples can be explained by the higher cut off values used when analyzing the HIV cohort. The cut-off value varied due to varying background signal in the different experiments, a problem that has been minimized in the final RT load assay: ExaVir^{TM}. The sensitivity of current RT load procedure could be further improved by using more plasma or by prolonging RT reaction duration.

5.4.2 Phenotypic drug susceptibility testing based on HIV-1 RT recovered from human plasma (V)

Drug resistance tests aim to identify the failing drugs. The established tests have limitations for use as they are, for example, either labor intensive and time consuming or have difficulty in interpretation of the results (see 3.2.2). It is essential to develop simple, rapid, and reliable methods for drug susceptibility analysis. The RT load procedure successfully purifies RT from virions in plasma and removes disturbing factors. RT derived directly from human plasma is thereby available for drug susceptibility testing.

5.4.2.1 Optimization of the drug susceptibility tests

Susceptibility to NNRTIs was determined at saturated dNTP substrate concentrations supporting a DNA elongation reaction close to maximal velocity. Thus the assay requires comparatively small amounts of RT.

The thymidine (T)-analogue drug susceptibility assay was based on a compromise between the conditions required for a rapid DNA elongation reaction and ability to distinguish between resistant and sensitive RT enzymes, with respect to chain terminators. The RT reaction mixture used must be able to support an energy dependent ddNMP excision reaction. Many factors like ionic strength, pH, concentration of dNTP substrate, primer amount and ATP concentration affect this reaction and were optimized experimentally. As seen in Fig.7, when the reaction solution was adjusted to pH7.0 the highest fold change in IC_{50} value was obtained for recombinant mutant RT with aa substitutions M41L, T69S-SS, L210W and T215Y. A further decrease in pH value led to a dramatic reduction in RT activity.

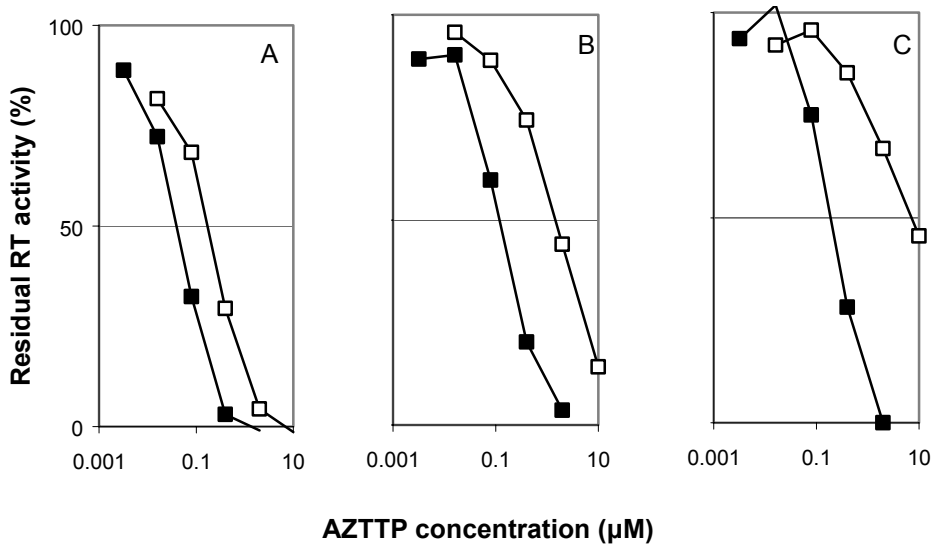

Fig. 7. Influence of pH on capacity of the T analogue susceptibility test to distinguish AZT resistant RT (□) from the sensitive RT (■). The pH values tested were: 7.6 (A), 7.3 (B) and 7.0 (C).

To evaluate the usefulness of the test, recombinant RTs with aa substitutions in a defined wild type background were investigated. They gave in general the expected drug susceptibility pattern (Table 1, Paper V). It is interesting to note that there was a span in IC_{50} values among three wild type RTs tested. This might be due to differences in, for example, the structure and purity of RT occurred during RT preparation. Therefore, it should be avoided to compare the results obtained with recombinant enzymes from different sources.

5.4.2.2 Susceptibility to NNRTIs of HIV-1 RT recovered from patient plasma

Clear difference was observed for the susceptibility of the plasma RTs to all NNRTIs tested (Table 3, Paper V). Plasma RTs that had no drug resistance associated aa substitutions gave similar IC_{50} values, while those containing aa substitutions associated with resistance to NNRTIs had IC_{50} values larger than the highest drug concentration tested for at least two of the NNRTIs drugs. RT 3236, which was resistant to NVP and EFV, exhibited an increased sensitivity to DLV and carried the substitutions A98G and G190A. This was not unexpected since the G190A substitution has earlier been connected to hypersensitivity to DLV (Huang et al., 2000). RT 656 and 3850, which contained the same NNRTI associated substitutions, A98G and Y181C, were highly resistant to NVP and DLV. However, they exhibited significantly different susceptibilities to EFV (Table 3. Paper V). This may be the result of interactions with other unknown substitutions as both RTs had substitutions associated with resistance to NRTIs (Table 4, Paper V). In addition, sample 656 also contained much more RNA copies than sample 3850, which indicated that sample 656 might be more resistant to EFV. In search of viruses carrying A98G and Y181C in combination with miscellaneous NRTI mutations in their RT genes in Stanford University's database http://hivdb.stanford.edu/cgi-bin/RT_Phenotype.cgi, a variation from 1.0 to 135 in fold resistance to EFV in cell culture was observed. Such a difference can hardly be predicted by genotypic drug resistance tests, which demonstrates the advantage of the RT based phenotypic drug susceptibility assay when compared to genotypic testing. The results were in accordance with genotypic data. Interestingly, the span of IC_{50} values obtained in this RT assay was in the same range as that found in cell culture (see also http://hivdb.stanford.edu).

5.4.2.3 Susceptibility to AZT-TP and d4T-TP of HIV-1 RT recovered from patient plasma

As in NNRTI susceptibility assay, similar IC_{50} values were obtained for RTs considered wild type based on their RT gene sequences (Table 4, Paper V). The highest fold increase, relative to the average IC_{50} for wild type plasma RTs, was 1.5 for AZT-TP and 1.4 for d4T-TP. Samples having aa substitutions known to give NRTI resistance were distinguishable and had 2.2-30 fold increased IC_{50} values.

Five RTs had intermediate range of fold increase in IC_{50} values. They carried multiple NRTI resistance associated aa substitutions and, in addition, at least one of the substitutions M184V or Y181C which are known to cause re-sensitization to T analogue drugs (Boucher et al., 1993; Gotte et al., 2000; Larder, 1992; Larder, 1994; Vandamme et al., 1998). Among RTs that exhibited highly elevated IC_{50} values to both AZT-TP and d4T-TP, RT 393 had a set of substitutions representative for the Q151M complex (Shirasaka et al., 1995), while RT 1859 contained the T69E-G insertion, in addition to the M41L, E44D, D67N, L210W, T215Y and V118I substitutions (Table 4, Paper V)). The combination of insertions at aa 69 and other

AZT resistance mutations have been found to cause high-level resistance to all NRTIs (Larder et al., 1999; Winters et al., 1998).

To summarize, the phenotypic drug sensitivity profiles of the RTs were concordant with the patterns of drug resistance mutations. The assays can thus be useful for analysis of HIV-1 susceptibility towards NNRTIs and T analogue drugs.

6. GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis a set of sensitive non-radioactive RT assays were developed and applied for different purposes. They are: standard RT inhibition assay; RT binding inhibition assay; RT-protein detection assay; and chain termination or template-primer destruction assay.

When analyzed in the presented colorimetric solid-phase assays, different types of inhibitors exhibited different behaviors in relation to their mechanism of action. The combined use of the assays could dissect the mechanism of action of RT inhibitors. The study of four NNRTIs demonstrated that none of them prevented RT binding to template-primer, and that MSA-300 had a higher affinity for RT than NVP, EFV and DLV. The IC_{50} values obtained were similar to those reported for the soluble RT assays, which indicated that the assays could be used for screening of RT inhibitory substances.

The CT assay and a modified IC assay were used for characterization of 18 HIV-1 cell culture isolates having various susceptibilities to AZT. CT_{50} values had a tendency to increase with the occurrence of more AZT resistance associated mutations in the RT gene. A more than 600-fold difference in CT_{50} value was observed between the most resistant and most sensitive isolates. The usefulness of the CT assay for characterization of AZT resistance needs to be further investigated with site directed mutants originating from the same wild type virus.

An RT load procedure (ExaVir^{TM} Load) was developed to measure and characterize HIV-1 RT from plasma. The RT load showed a strong correlation with RNA values in a study of 391 samples from HIV positive patients. The assay was able to detect a broad range of different HIV subtypes.

Two drug susceptibility tests, based on RT derived directly from plasma, were developed for NNRTIs or T analogue drugs, respectively. A high degree of concordance was found between the drug susceptibility profiles of plasma RTs and the occurrence of mutations associated with drug resistance. The assays are technically simple, rapid, affordable, and do not require complex interpretation of the results. They provide interesting alternatives for analyzing phenotypic drug susceptibility especially when the therapy is based on NNRTIs and T analogue drugs.

The current assay system for measuring of RT activity, screening of anti-HIV drugs and dissection of the mechanism of their action is useful for other retroviruses, with a modification of the reaction conditions. The principle of measuring RT in plasma and determination of drug susceptibility may also be applied to other enzymes that are protected by virions.

To broaden the use of the current assays, we have made microtitre plates with bound DNA-template containing all four nucleotides. This plate can be used to measure inhibition of RT by all types of NRTIs and NNRTIs (Lennerstrand et al., 2002). The sensitivity of this prototype is currently a little too low for drug susceptibility testing in general, but it is sufficient for characterization of RT from e.g. primary culture of cassette virus and patient plasma holding high viral load. Such an assay also provides an alternative approach for analysis of the mechanism of action of RT inhibitors, as it measures DNA dependent DNA polymerase activity of RT.

7. ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all who made a contribution to this work, especially to:
Simon Gronowitz, my supervisor, for creative ideas, enormous supports, patient teachings consciously and unconsciously (when talking to the other people), for warm parties, sea-fishings and museum-visitings, for sharing his interesting family stories back in both the west and the east of China. Simon tells me 'no problem' whenever I get worried. **Stina** and her family, for interesting talks and nice time together.
Clas Källander, my supervisor, for endless support, great ideas and patience, for carefully reading and commenting on every sentence that I have written, for successfully getting an 'E' permission for me, for taking all computer related problems, and for letting me feel that I owe him too much.
Professor **Eva Maria Fenyö**, my supervisor, for enthusiastic support, encouragement and great care, for leading me through the process of PhD study and making my dream close to reality.
Professor **Bo Öberg**, my co-supervisor, for introducing me into Karolinska Institute.
Johan Lennerstrand, my co-supervisor, for strong support with his knowledge in science, for discussions and collaborations, and for prompt answers to all questions.
Mikael Rhen and Yihai Cao, for being my mentors.
Ingvar Pettersson, for enthusiastic support and gentle supervision, which makes work more enjoyable.
Gary Corrigan, for great fun and support, for teaching me Irish, patiently reading all my publications and this book and giving excellent comments both about science and my Chin-english, for our happy song 'sga b-don b-don' and great place 'ho-chi city', 'it is nice to see you to see you nice'.
Anders Malmsten, for enthusiasm, all time support and my special birthday party, for bringing me close to the history, culture and society of Sweden, above all, for going through ups and downs together. **Solveig**, for being so warm-hearted and playing with little Shuai.
Ann-sofie Rytting, for encouragement and fruitful collaboration, for interesting discussions and meals, for always trying to help my family and me.
Henric Ekstrand, for fruitful collaboration and helping me doing my first experiment in Sweden, for support and trust.
Lars Skoglund, Kurt Rydé, Göran Pettersson and Thomas Pollare, for understanding and support that they may not realize the importance of. **Tommy Gatu**, for productive productions, advice for schools, and jokings. **Andreas Pettersson**, for quiet support and handing out money. **Staffan Sjödahl**, for conversations with no limits, and making working in the lab more interesting. **Kajsa Löwenhielm**, for support, teaching me drawing pictures and keeping the secret. **Nils-Fredrik Kaiser**, for sharing a room and discussions about food and Taiwan. **Tobias Gronowitz, Therese Lindgren, Maria Andersson, Maria Sjöstedt, Ina Hubatsch, Ulla Långström-Persson**, for support inside and outside job, and for making Cavidi a happy place to work at. **Juxian Zheng**, for great support.
My co-authors: **Sandra Hjalmarsson, Lotta Vrang, Bo Svennerholm, Jonas Blomberg, Anders Sönnerborg, Kajsa Aperia, Eric Sandström, Tomas Leitner, Torsten Unge, and Rama Bhikhabhai.** It couldn't have been done without you!
Hong Zhang, for discussion and materials. **Raymond Awad**, for fun and exchange between cultures of thousands years of age. **Anna Lögdberg** and **Cecilia Danielson**, for great help and patience.

Professors **Peizhen Tao** and **Hongshan Chen**, my supervisors in China, for support, care, and for introducing me into the antiviral world.
My **Chinese friends** in Uppsala, for making my life in Sweden more colorful.
My **parents**, my **aunt** and **uncles**, my **sisters** and **brother**, and my **grandparents**, for love, support, and best wishes.
Shusheng, for being in the same boat of life, for understanding, encouragement and support, and for constructive discussions in science.
Shuai, my son, for love and making my life happier and more meaningful!

8. REFERENCES

Aldovini, A., and Young, R. A. (1990): Mutations of RNA and protein sequences involved in human immunodeficiency virus type 1 packaging result in production of noninfectious virus. *J Virol* **64**, 1920-6.

Archer, R. H., Dykes, C., Gerondelis, P., Lloyd, A., Fay, P., Reichman, R. C., Bambara, R. A., and Demeter, L. M. (2000): Mutants of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase resistant to nonnucleoside reverse transcriptase inhibitors demonstrate altered rates of RNase H cleavage that correlate with HIV-1 replication fitness in cell culture. *J Virol* **74**, 8390-401.

Arion, D., Kaushik, N., McCormick, S., Borkow, G., and Parniak, M. A. (1998): Phenotypic mechanism of HIV-1 resistance to 3'-azido-3'-deoxythymidine (AZT): increased polymerization processivity and enhanced sensitivity to pyrophosphate of the mutant viral reverse transcriptase. *Biochemistry* **37**, 15908-17.

Arnold, E., Jacobo-Molina, A., Nanni, R. G., Williams, R. L., Lu, X., Ding, J., Clark, A. D., Jr., Zhang, A., Ferris, A. L., Clark, P., Hizi, A., and Hughes, S. H. (1992): Structure of HIV-1 reverse transcriptase/DNA complex at 7 A resolution showing active site locations. *Nature* **357**, 85-9.

Arts, E. J., Quinones-Mateu, M. E., Albright, J. L., Marois, J. P., Hough, C., Gu, Z., and Wainberg, M. A. (1998): 3'-Azido-3'-deoxythymidine (AZT) mediates cross-resistance to nucleoside analogs in the case of AZT-resistant human immunodeficiency virus type 1 variants. *J Virol* **72**, 4858-65.

Baltimore, D. (1970): RNA-dependent DNA polymerase in virions of RNA tumour viruses. *Nature* **226**, 1209-11.

Balzarini, J. (1999): Suppression of resistance to drugs targeted to human immunodeficiency virus reverse transcriptase by combination therapy. *Biochem Pharmacol* **58**, 1-27.

Barat, C., Lullien, V., Schatz, O., Keith, G., Nugeyre, M. T., Gruninger-Leitch, F., Barre-Sinoussi, F., LeGrice, S. F., and Darlix, J. L. (1989): HIV-1 reverse transcriptase specifically interacts with the anticodon domain of its cognate primer tRNA. *Embo J* **8**, 3279-85.

Barre-Sinoussi, F., Chermann, J. C., Rey, F., Nugeyre, M. T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., Rozenbaum, W., and Montagnier, L. (1983): Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* **220**, 868-71.

Battula, N., and Loeb, L. A. (1974): The infidelity of avian myeloblastosis virus deoxyribonucleic acid polymerase in polynucleotide replication. *J Biol Chem* **249**, 4086-93.

Battula, N., and Loeb, L. A. (1976): On the fidelity of DNA replication. Lack of exodeoxyribonuclease activity and error-correcting function in avian myeloblastosis virus DNA polymerase. *J Biol Chem* **251**, 982-6.

Boucher, C. A., Cammack, N., Schipper, P., Schuurman, R., Rouse, P., Wainberg, M. A., and Cameron, J. M. (1993): High-level resistance to (-) enantiomeric 2'-deoxy-3'-thiacytidine in vitro is due to one amino acid substitution in the catalytic site of human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob Agents Chemother* **37**, 2231-4.

Boyer, J. C., Bebenek, K., and Kunkel, T. A. (1992): Unequal human immunodeficiency virus type 1 reverse transcriptase error rates with RNA and DNA templates. *Proc Natl Acad Sci U S A* **89**, 6919-23.

Boyer, P. L., Sarafianos, S. G., Arnold, E., and Hughes, S. H. (2001): Selective excision of AZTMP by drug-resistant human immunodeficiency virus reverse transcriptase. *J Virol* **75**, 4832-42.

Braaten, D., Franke, E. K., and Luban, J. (1996): Cyclophilin A is required for the replication of group M human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus SIV(CPZ)GAB but not group O HIV-1 or other primate immunodeficiency viruses. *J Virol* **70**, 4220-7.

Braun, J., Plantier, J. C., Hellot, M. F., Tuaillon, E., Gueudin, M., Damond, F., Malmsten, A., Corrigan, G. E., and Simon, F. (2003): A new quantitative HIV load assay based on plasma virion reverse transcriptase activity for the different types, groups and subtypes. *Aids* **17**, 331-336.

Bryant, M., and Ratner, L. (1990): Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. *Proc Natl Acad Sci U S A* **87**, 523-7.

Caliendo, A. M., Savara, A., An, D., DeVore, K., Kaplan, J. C., and D'Aquila, R. T. (1996): Effects of zidovudine-selected human immunodeficiency virus type 1 reverse transcriptase amino acid substitutions on processive DNA synthesis and viral replication. *J Virol* **70**, 2146-53.

Carpenter, C. C., Fischl, M. A., Hammer, S. M., Hirsch, M. S., Jacobsen, D. M., Katzenstein, D. A., Montaner, J. S., Richman, D. D., Saag, M. S., Schooley, R. T., Thompson, M. A., Vella, S., Yeni, P. G., and Volberding, P. A. (1998): Antiretroviral therapy for HIV infection in 1998: updated recommendations of the International AIDS Society-USA Panel. *Jama* **280**, 78-86.

Carroll, S. S., Geib, J., Olsen, D. B., Stahlhut, M., Shafer, J. A., and Kuo, L. C. (1994): Sensitivity of HIV-1 reverse transcriptase and its mutants to inhibition by azidothymidine triphosphate. *Biochemistry* **33**, 2113-20.

Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P. D., Wu, L., Mackay, C. R., LaRosa, G., Newman, W., Gerard, N., Gerard, C., and Sodroski, J. (1996): The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* **85**, 1135-48.

Clark, S. J., Saag, M. S., Decker, W. D., Campbell-Hill, S., Roberson, J. L., Veldkamp, P. J., Kappes, J. C., Hahn, B. H., and Shaw, G. M. (1991): High titers of cytopathic virus in plasma of patients with symptomatic primary HIV-1 infection. *N Engl J Med* **324**, 954-60.

Clavel, F., Guetard, D., Brun-Vezinet, F., Chamaret, S., Rey, M. A., Santos-Ferreira, M. O., Laurent, A. G., Dauguet, C., Katlama, C., Rouzioux, C., Klatzmann, D., Champalimaud, J. L., and Montagnier, L. (1986): Isolation of a new human retrovirus from West African patients with AIDS. *Science* **233**, 343-6.

Coffin, J., Haase, A., Levy, J. A., Montagnier, L., Oroszlan, S., Teich, N., Temin, H., Toyoshima, K., Varmus, H., Vogt, P., and Weiss, R. (1986): Human immunodeficiency viruses. *Science* **232**, 697.

Coffin, J. M. (1995): HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. *Science* **267**, 483-9.

Collins, K. L., Chen, B. K., Kalams, S. A., Walker, B. D., and Baltimore, D. (1998): HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. *Nature* **391**, 397-401.

Conway, B., Wainberg, M. A., Hall, D., Harris, M., Reiss, P., Cooper, D., Vella, S., Curry, R., Robinson, P., Lange, J. M., and Montaner, J. S. (2001): Development of drug resistance in patients receiving combinations of zidovudine, didanosine and nevirapine. *Aids* **15**, 1269-74.

Coombs, R. W., Collier, A. C., Allain, J. P., Nikora, B., Leuther, M., Gjerset, G. F., and Corey, L. (1989): Plasma viremia in human immunodeficiency virus infection. *N Engl J Med* **321**, 1626-31.

Corrigan, G. E., Al-Khalili, L., Malmsten, A., Thorstensson, R., Fenyo, E. M., Kallander, C. F., and Gronowitz, J. S. (1998): Differences in reverse transcriptase activity versus p24 antigen detection in cell culture, when comparing a homogeneous group of HIV type 1 subtype B viruses with a heterogeneous group of divergent strains. *AIDS Res Hum Retroviruses* **14**, 347-52.

Daar, E. S., Moudgil, T., Meyer, R. D., and Ho, D. D. (1991): Transient high levels of viremia in patients with primary human immunodeficiency virus type 1 infection. *N Engl J Med* **324**, 961-4.

Davey, R. T., Jr., Dewar, R. L., Reed, G. F., Vasudevachari, M. B., Polis, M. A., Kovacs, J. A., Falloon, J., Walker, R. E., Masur, H., Haneiwich, S. E., O'Neill, D. G., Decker, M. R., Metcalf, J. A., Deloria, M. A., Laskin, O. L., Salzman, N., and Lane, H. C. (1993): Plasma viremia as a sensitive indicator of the antiretroviral activity of L-697,661. *Proc Natl Acad Sci U S A* **90**, 5608-12.

De Clercq, E. (1998): The role of non-nucleoside reverse transcriptase inhibitors (NNRTIs) in the therapy of HIV-1 infection. *Antiviral Res* **38**, 153-79.

DeGruttola, V., Beckett, L. A., Coombs, R. W., Arduino, J. M., Balfour, H. H., Jr., Rasheed, S., Hollinger, F. B., Fischl, M. A., and Volberding, P. (1994): Serum p24 antigen level as an intermediate end point in clinical trials of zidovudine in people infected with human immunodeficiency virus type 1. Aids Clinical Trials Group Virology Laboratories. *J Infect Dis* **169**, 713-21.

Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R. E., Hill, C. M., Davis, C. B., Peiper, S. C., Schall, T. J., Littman, D. R., and Landau, N. R. (1996): Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **381**, 661-6.

Descamps, D., Collin, G., Letourneur, F., Apetrei, C., Damond, F., Loussert-Ajaka, I., Simon, F., Saragosti, S., and Brun-Vezinet, F. (1997): Susceptibility of human immunodeficiency virus type 1 group O isolates to antiretroviral agents: in vitro phenotypic and genotypic analyses. *J Virol* **71**, 8893-8.

Ding, J., Das, K., Moereels, H., Koymans, L., Andries, K., Janssen, P. A., Hughes, S. H., and Arnold, E. (1995): Structure of HIV-1 RT/TIBO R 86183 complex reveals similarity in the binding of diverse nonnucleoside inhibitors. *Nat Struct Biol* **2**, 407-15.

Doranz, B. J., Rucker, J., Yi, Y., Smyth, R. J., Samson, M., Peiper, S. C., Parmentier, M., Collman, R. G., and Doms, R. W. (1996): A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* **85**, 1149-58.

Dorfman, T., Bukovsky, A., Ohagen, A., Hoglund, S., and Gottlinger, H. G. (1994): Functional domains of the capsid protein of human immunodeficiency virus type 1. *J Virol* **68**, 8180-7.

Eberle, J., and Seibl, R. (1992): A new method for measuring reverse transcriptase activity by ELISA. *J Virol Methods* **40**, 347-56.

Ekstrand, D. H., Awad, R. J., Källander, C. F. R., and Gronowitz, J. S. (1996): A sensitive assay for the quantification of reverse transcriptase activity based on the use of carrier-bound template and non-radioactive-product detection, with special reference to human-immunodeficiency-virus isolation. *Biotechnol Appl Biochem* **23(Pt 2)**, 95-105.

Eron, J. J., Benoit, S. L., Jemsek, J., MacArthur, R. D., Santana, J., Quinn, J. B., Kuritzkes, D. R., Fallon, M. A., and Rubin, M. (1995): Treatment with lamivudine, zidovudine,

or both in HIV-positive patients with 200 to 500 CD4+ cells per cubic millimeter. North American HIV Working Party. *N Engl J Med* **333**, 1662-9.

Eron, J. J., Chow, Y. K., Caliendo, A. M., Videler, J., Devore, K. M., Cooley, T. P., Liebman, H. A., Kaplan, J. C., Hirsch, M. S., and D'Aquila, R. T. (1993): pol mutations conferring zidovudine and didanosine resistance with different effects in vitro yield multiply resistant human immunodeficiency virus type 1 isolates in vivo. *Antimicrob Agents Chemother* **37**, 1480-7.

Esnouf, R., Ren, J., Ross, C., Jones, Y., Stammers, D., and Stuart, D. (1995): Mechanism of inhibition of HIV-1 reverse transcriptase by non-nucleoside inhibitors. *Nat Struct Biol* **2**, 303-8.

Fan, N., Rank, K. B., Evans, D. B., Thomas, R. C., Tarpley, W. G., and Sharma, S. K. (1995): Simultaneous mutations at Tyr-181 and Tyr-188 in HIV-1 reverse transcriptase prevents inhibition of RNA-dependent DNA polymerase activity by the bisheteroarylpiperazine (BHAP) U-90152s. *FEBS Lett* **370**, 59-62.

Farnet, C. M., and Haseltine, W. A. (1990): Integration of human immunodeficiency virus type 1 DNA in vitro. *Proc Natl Acad Sci U S A* **87**, 4164-8.

Fauci, A. S., and Lane, H. C. (1991): The acquired immunodeficiency syndrome (AIDS). In Harrison's principles of internal medicine, 12th edition (ed. J.D. Wilson et al.), pp. 1402-1410. McGraw-Hill, New York.

Feng, Y., Broder, C. C., Kennedy, P. E., and Berger, E. A. (1996): HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272**, 872-7.

Flexner, C. (1998): HIV-protease inhibitors. *N Engl J Med* **338**, 1281-92.

Fornerod, M., van Deursen, J., van Baal, S., Reynolds, A., Davis, D., Murti, K. G., Fransen, J., and Grosveld, G. (1997): The human homologue of yeast CRM1 is in a dynamic subcomplex with CAN/Nup214 and a novel nuclear pore component Nup88. *Embo J* **16**, 807-16.

Fouchier, R. A., Meyer, B. E., Simon, J. H., Fischer, U., and Malim, M. H. (1997): HIV-1 infection of non-dividing cells: evidence that the amino-terminal basic region of the viral matrix protein is important for Gag processing but not for post-entry nuclear import. *Embo J* **16**, 4531-9.

Freed, E. O. (1998): HIV-1 gag proteins: diverse functions in the virus life cycle. *Virology* **251**, 1-15.

Gaines, H., von Sydow, M. A., von Stedingk, L. V., Biberfeld, G., Bottiger, B., Hansson, L. O., Lundbergh, P., Sonnerborg, A. B., Wasserman, J., and Strannegaard, O. O. (1990): Immunological changes in primary HIV-1 infection. *Aids* **4**, 995-9.

Gallo, R. C., Salahuddin, S. Z., Popovic, M., Shearer, G. M., Kaplan, M., Haynes, B. F., Palker, T. J., Redfield, R., Oleske, J., Safai, B., White, G., and Markham, P. D. (1984): Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science* **224**, 500-3.

Garcia Lerma, J. G., Yamamoto, S., Gomez-Cano, M., Soriano, V., Green, T. A., Busch, M. P., Folks, T. M., and Heneine, W. (1998): Measurement of human immunodeficiency virus type 1 plasma virus load based on reverse transcriptase (RT) activity: evidence of variabilities in levels of virion-associated RT. *J Infect Dis* **177**, 1221-9.

Gerondelis, P., Archer, R. H., Palaniappan, C., Reichman, R. C., Fay, P. J., Bambara, R. A., and Demeter, L. M. (1999): The P236L delavirdine-resistant human immunodeficiency virus type 1 mutant is replication defective and demonstrates alterations in both RNA 5'-end- and DNA 3'-end-directed RNase H activities. *J Virol* **73**, 5803-13.

Gnann, J. W., Jr., Nelson, J. A., and Oldstone, M. B. (1987): Fine mapping of an immunodominant domain in the transmembrane glycoprotein of human immunodeficiency virus. *J Virol* **61**, 2639-41.

Goff, S., Traktman, P., and Baltimore, D. (1981): Isolation and properties of Moloney murine leukemia virus mutants: use of a rapid assay for release of virion reverse transcriptase. *J Virol* **38**, 239-48.

Gotte, M., Arion, D., Parniak, M. A., and Wainberg, M. A. (2000): The M184V mutation in the reverse transcriptase of human immunodeficiency virus type 1 impairs rescue of chain-terminated DNA synthesis. *J Virol* **74**, 3579-85.

Gotte, M., Li, X., and Wainberg, M. A. (1999): HIV-1 reverse transcription: a brief overview focused on structure-function relationships among molecules involved in initiation of the reaction. *Arch Biochem Biophys* **365**, 199-210.

Gottlinger, H. G., Sodroski, J. G., and Haseltine, W. A. (1989): Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. *Proc Natl Acad Sci U S A* **86**, 5781-5.

Greene, W. C. (1991): The molecular biology of human immunodeficiency virus type 1 infection. *N Engl J Med* **324**, 308-17.

Gronowitz, J. S., Lennerstrand, J., Pettersson, A., Neumuller, M., Johansson, M., and Källander, C. F. R. (1992): Determination of IC50 values and mechanism of action of HIV1 RT inhibitors, by the use of carrierbound template-primer, template, or primer, with 125I-IUTP as substrate. *Antivir Chem Chemother* **3**, 203-213.

Gronowitz, J. S., Neumuller, M., Lennerstrand, J., Bhikhabhai, R., Unge, T., Weltman, H., and Källander, C. F. (1991): Carrier bound templates for single tube reverse transcriptase assays and for combined purification and activity analyses, with special reference to HIV. *Biotechnol Appl Biochem* **13**, 127-42.

Gu, Z., Salomon, H., Cherrington, J. M., Mulato, A. S., Chen, M. S., Yarchoan, R., Foli, A., Sogocio, K. M., and Wainberg, M. A. (1995): K65R mutation of human immunodeficiency virus type 1 reverse transcriptase encodes cross-resistance to 9-(2-phosphonylmethoxyethyl)adenine. *Antimicrob Agents Chemother* **39**, 1888-91.

Gulick, R. M., Mellors, J. W., Havlir, D., Eron, J. J., Gonzalez, C., McMahon, D., Jonas, L., Meibohm, A., Holder, D., Schleif, W. A., Condra, J. H., Emini, E. A., Isaacs, R., Chodakewitz, J. A., and Richman, D. D. (1998): Simultaneous vs sequential initiation of therapy with indinavir, zidovudine, and lamivudine for HIV-1 infection: 100-week follow-up. *Jama* **280**, 35-41.

Gurtler, L. G., Hauser, P. H., Eberle, J., von Brunn, A., Knapp, S., Zekeng, L., Tsague, J. M., and Kaptue, L. (1994): A new subtype of human immunodeficiency virus type 1 (MVP-5180) from Cameroon. *J Virol* **68**, 1581-5.

Hammer, S. M., Katzenstein, D. A., Hughes, M. D., Gundacker, H., Schooley, R. T., Haubrich, R. H., Henry, W. K., Lederman, M. M., Phair, J. P., Niu, M., Hirsch, M. S., and Merigan, T. C. (1996): A trial comparing nucleoside monotherapy with combination therapy in HIV-infected adults with CD4 cell counts from 200 to 500 per cubic millimeter. AIDS Clinical Trials Group Study 175 Study Team. *N Engl J Med* **335**, 1081-90.

Hammer, S. M., Squires, K. E., Hughes, M. D., Grimes, J. M., Demeter, L. M., Currier, J. S., Eron, J. J., Jr., Feinberg, J. E., Balfour, H. H., Jr., Deyton, L. R., Chodakewitz, J. A., and Fischl, M. A. (1997): A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. AIDS Clinical Trials Group 320 Study Team. *N Engl J Med* **337**, 725-33.

Hanna, G. J., and D'Aquila, R. T. (1999): Antiretroviral Drug Resistance in HIV-1. *Curr Infect Dis Rep* **1**, 289-297.

Harrigan, P. R., Kinghorn, I., Bloor, S., Kemp, S. D., Najera, I., Kohli, A., and Larder, B. A. (1996): Significance of amino acid variation at human immunodeficiency virus type 1 reverse transcriptase residue 210 for zidovudine susceptibility. *J Virol* **70**, 5930-4.

Havlir, D. V., Eastman, S., Gamst, A., and Richman, D. D. (1996): Nevirapine-resistant human immunodeficiency virus: kinetics of replication and estimated prevalence in untreated patients. *J Virol* **70**, 7894-9.

Heneine, W., Yamamoto, S., Switzer, W. M., Spira, T. J., and Folks, T. M. (1995): Detection of reverse transcriptase by a highly sensitive assay in sera from persons infected with human immunodeficiency virus type 1. *J Infect Dis* **171**, 1210-6.

Hertogs, K., de Bethune, M. P., Miller, V., Ivens, T., Schel, P., Van Cauwenberge, A., Van Den Eynde, C., Van Gerwen, V., Azijn, H., Van Houtte, M., Peeters, F., Staszewski, S., Conant, M., Bloor, S., Kemp, S., Larder, B., and Pauwels, R. (1998): A rapid method for simultaneous detection of phenotypic resistance to inhibitors of protease and reverse transcriptase in recombinant human immunodeficiency virus type 1 isolates from patients treated with antiretroviral drugs. *Antimicrob Agents Chemother* **42**, 269-76.

Hizi, A., Shaharabany, M., Tal, R., and Hughes, S. H. (1992): The effects of cysteine mutations on the reverse transcriptases of human immunodeficiency virus types 1 and 2. *J Biol Chem* **267**, 1293-7.

Hizi, A., Tal, R., Shaharabany, M., Currens, M. J., Boyd, M. R., Hughes, S. H., and McMahon, J. B. (1993): Specific inhibition of the reverse transcriptase of human immunodeficiency virus type 1 and the chimeric enzymes of human immunodeficiency virus type 1 and type 2 by nonnucleoside inhibitors. *Antimicrob Agents Chemother* **37**, 1037-42.

Ho, D. D., Moudgil, T., and Alam, M. (1989): Quantitation of human immunodeficiency virus type 1 in the blood of infected persons. *N Engl J Med* **321**, 1621-5.

Ho, D. D., Neumann, A. U., Perelson, A. S., Chen, W., Leonard, J. M., and Markowitz, M. (1995): Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* **373**, 123-6.

Hooker, D. J., Tachedjian, G., Solomon, A. E., Gurusinghe, A. D., Land, S., Birch, C., Anderson, J. L., Roy, B. M., Arnold, E., and Deacon, N. J. (1996): An in vivo mutation from leucine to tryptophan at position 210 in human immunodeficiency virus type 1 reverse transcriptase contributes to high-level resistance to 3'-azido-3'-deoxythymidine. *J Virol* **70**, 8010-8.

Hostomsky, Z., Hostomska, Z., Fu, T. B., and Taylor, J. (1992): Reverse transcriptase of human immunodeficiency virus type 1: functionality of subunits of the heterodimer in DNA synthesis. *J Virol* **66**, 3179-82.

Hsieh, J. C., Zinnen, S., and Modrich, P. (1993): Kinetic mechanism of the DNA-dependent DNA polymerase activity of human immunodeficiency virus reverse transcriptase. *J Biol Chem* **268**, 24607-13.

Hsiou, Y., Ding, J., Das, K., Clark, A. D., Jr., Boyer, P. L., Lewi, P., Janssen, P. A., Kleim, J. P., Rosner, M., Hughes, S. H., and Arnold, E. (2001): The Lys103Asn mutation of HIV-1 RT: a novel mechanism of drug resistance. *J Mol Biol* **309**, 437-45.

Huang, H., Chopra, R., Verdine, G. L., and Harrison, S. C. (1998): Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. *Science* **282**, 1669-75.

Huang, W., Garmarnik, A., and Werin, T. (2000): NNRTI-resistance profiles, replicative capacity and protease processing defects in HIV-1 that contain RT mutations at amino acid 190. *Antivir Ther* **5**(Supplement 3), 23.

Hubscher, U., Nasheuer, H. P., and Syvaoja, J. E. (2000): Eukaryotic DNA polymerases, a growing family. *Trends Biochem Sci* **25**, 143-7.

Jacobo-Molina, A., Clark, A. D., Jr., Williams, R. L., Nanni, R. G., Clark, P., Ferris, A. L., Hughes, S. H., and Arnold, E. (1991): Crystals of a ternary complex of human immunodeficiency virus type 1 reverse transcriptase with a monoclonal antibody Fab fragment and double-stranded DNA diffract x-rays to 3.5-A resolution. *Proc Natl Acad Sci U S A* **88**, 10895-9.

Jacobo-Molina, A., Ding, J., Nanni, R. G., Clark, A. D., Jr., Lu, X., Tantillo, C., Williams, R. L., Kamer, G., Ferris, A. L., Clark, P., Hizi, A., Hughes, S. H., and Arnold, E. (1993): Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 A resolution shows bent DNA. *Proc Natl Acad Sci U S A* **90**, 6320-4.

Japour, A. J., Mayers, D. L., Johnson, V. A., Kuritzkes, D. R., Beckett, L. A., Arduino, J. M., Lane, J., Black, R. J., Reichelderfer, P. S., D'Aquila, R. T., and Crumpacker, C. S. (1993): Standardized peripheral blood mononuclear cell culture assay for determination of drug susceptibilities of clinical human immunodeficiency virus type 1 isolates. The RV-43 Study Group, the AIDS Clinical Trials Group Virology Committee Resistance Working Group. *Antimicrob Agents Chemother* **37**, 1095-101.

Jurriaans, S., Weverling, G. J., Goudsmit, J., Boogaard, J., Brok, M., Van Strijp, D., Lange, J., Koot, M., and Van Gemen, B. (1995): Distinct changes in HIV type 1 RNA versus p24 antigen levels in serum during short-term zidovudine therapy in asymptomatic individuals with and without progression to AIDS. *AIDS Res Hum Retroviruses* **11**, 473-9.

Kanki, P. J., Travers, K. U., MBoups, S., Hsieh, C. C., Marlink, R. G., Gueye, N. A., Siby, T., Thior, I., Hernandez-Avila, M., Sankale, J. L., NDoye, I., and Essex, M. E. (1994): Slower heterosexual spread of HIV-2 than HIV-1. *Lancet* **343**, 943-6.

Kati, W. M., Johnson, K. A., Jerva, L. F., and Anderson, K. S. (1992): Mechanism and fidelity of HIV reverse transcriptase. *J Biol Chem* **267**, 25988-97.

Kellam, P., Boucher, C. A., and Larder, B. A. (1992): Fifth mutation in human immunodeficiency virus type 1 reverse transcriptase contributes to the development of high-level resistance to zidovudine. *Proc Natl Acad Sci U S A* **89**, 1934-8.

Kellam, P., and Larder, B. A. (1994): Recombinant virus assay: a rapid, phenotypic assay for assessment of drug susceptibility of human immunodeficiency virus type 1 isolates. *Antimicrob Agents Chemother* **38**, 23-30.

Kleim, J. P., Bender, R., Kirsch, R., Meichsner, C., Paessens, A., and Riess, G. (1994): Mutational analysis of residue 190 of human immunodeficiency virus type 1 reverse transcriptase. *Virology* **200**, 696-701.

Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1992): Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* **256**, 1783-90.

Kostrikis, L. G., Bagdades, E., Cao, Y., Zhang, L., Dimitriou, D., and Ho, D. D. (1995): Genetic analysis of human immunodeficiency virus type 1 strains from patients in Cyprus: identification of a new subtype designated subtype I. *J Virol* **69**, 6122-30.

Kozal, M. J., Shah, N., Shen, N., Yang, R., Fucini, R., Merigan, T. C., Richman, D. D., Morris, D., Hubbell, E., Chee, M., and Gingeras, T. R. (1996): Extensive polymorphisms observed in HIV-1 clade B protease gene using high-density oligonucleotide arrays. *Nat Med* **2**, 753-9.

Lacey, S. F., Reardon, J. E., Furfine, E. S., Kunkel, T. A., Bebenek, K., Eckert, K. A., Kemp, S. D., and Larder, B. A. (1992): Biochemical studies on the reverse transcriptase and RNase H activities from human immunodeficiency virus strains resistant to 3'-azido-3'-deoxythymidine. *J Biol Chem* **267**, 15789-94.

Larder, B. A. (1992): 3'-Azido-3'-deoxythymidine resistance suppressed by a mutation conferring human immunodeficiency virus type 1 resistance to nonnucleoside reverse transcriptase inhibitors. *Antimicrob Agents Chemother* **36**, 2664-9.

Larder, B. A. (1994): Interactions between drug resistance mutations in human immunodeficiency virus type 1 reverse transcriptase. *J Gen Virol* **75 (Pt 5)**, 951-7.

Larder, B. A., Bloor, S., Kemp, S. D., Hertogs, K., Desmet, R. L., Miller, V., Sturmer, M., Staszewski, S., Ren, J., Stammers, D. K., Stuart, D. I., and Pauwels, R. (1999): A family of insertion mutations between codons 67 and 70 of human immunodeficiency virus type 1 reverse transcriptase confer multinucleoside analog resistance. *Antimicrob Agents Chemother* **43**, 1961-7.

Larder, B. A., Darby, G., and Richman, D. D. (1989): HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. *Science* **243**, 1731-4.

Larder, B. A., Kellam, P., and Kemp, S. D. (1991): Zidovudine resistance predicted by direct detection of mutations in DNA from HIV-infected lymphocytes. *Aids* **5**, 137-44.

Larder, B. A., and Kemp, S. D. (1989): Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). *Science* **246**, 1155-8.

Larder, B. A., and Stammers, D. K. (1999): Closing in on HIV drug resistance. *Nat Struct Biol* **6**, 103-6.

Law, M. G., Li, Y., McDonald, A. M., Cooper, D. A., and Kaldor, J. M. (2000): Estimating the population impact in Australia of improved antiretroviral treatment for HIV infection. *Aids* **14**, 197-201.

Le Grice, S. F., Naas, T., Wohlgensinger, B., and Schatz, O. (1991): Subunit-selective mutagenesis indicates minimal polymerase activity in heterodimer-associated p51 HIV-1 reverse transcriptase. *Embo J* **10**, 3905-11.

Lemp, G. F., Hirozawa, A. M., Cohen, J. B., Derish, P. A., McKinney, K. C., and Hernandez, S. R. (1992): Survival for women and men with AIDS. *J Infect Dis* **166**, 74-9.

Lemp, G. F., Payne, S. F., Neal, D., Temelso, T., and Rutherford, G. W. (1990a): Survival trends for patients with AIDS. *Jama* **263**, 402-6.

Lemp, G. F., Payne, S. F., Rutherford, G. W., Hessol, N. A., Winkelstein, W., Jr., Wiley, J. A., Moss, A. R., Chaisson, R. E., Chen, R. T., Feigal, D. W., Jr., and et al. (1990b): Projections of AIDS morbidity and mortality in San Francisco. *Jama* **263**, 1497-501.

Lennerstrand, J., Bergroth, T., Shao, X. W., Van Cauwenberge, A., Mc Kenna, P., and Sönnerborg, A. (2002): Cross-resistance patterns for HIV-1 muti-nucleoside resistant mutants with mutations associated with re-sensitization to Zidovudine. *Antivir Ther* **7**, 43.

Lennerstrand, J., Hertogs, K., Stammers, D. K., and Larder, B. A. (2001a): Correlation between viral resistance to zidovudine and resistance at the reverse transcriptase level for a panel of human immunodeficiency virus type 1 mutants. *J Virol* **75**, 7202-5.

Lennerstrand, J., Rytting, A. S., Vrang, L., Gronowitz, J. S., and Kallander, C. F. (1996): Application of a chain termination assay for characterisation of reverse transcriptase from AZT-resistant HIV isolates. *Antivir Chem Chemother* **7**, 313-320.

Lennerstrand, J., Stammers, D. K., and Larder, B. A. (2001b): Biochemical mechanism of human immunodeficiency virus type 1 reverse transcriptase resistance to stavudine. *Antimicrob Agents Chemother* **45**, 2144-6.

Lodge, R., Gottlinger, H., Gabuzda, D., Cohen, E. A., and Lemay, G. (1994): The intracytoplasmic domain of gp41 mediates polarized budding of human immunodeficiency virus type 1 in MDCK cells. *J Virol* **68**, 4857-61.

Louwagie, J., McCutchan, F. E., Peeters, M., Brennan, T. P., Sanders-Buell, E., Eddy, G. A., van der Groen, G., Fransen, K., Gershy-Damet, G. M., Deleys, R., and Burke, D. S. (1993): Phylogenetic analysis of gag genes from 70 international HIV-1 isolates provides evidence for multiple genotypes. *Aids* **7**, 769-80.

Loveday, C., and Tedder, R. S. (1993): Enzyme-linked immunosorbent assays for the measurement of human immunodeficiency virus, type 1 reverse transcriptase antigen and antibodies. *J Virol Methods* **41**, 181-92.

Markowitz, M. (2000): Resistance, fitness, adherence, and potency: mapping the paths to virologic failure. *Jama* **283**, 250-1.

Martinez-Picado, J., Sutton, L., De Pasquale, M. P., Savara, A. V., and D'Aquila, R. T. (1999): Human immunodeficiency virus type 1 cloning vectors for antiretroviral resistance testing. *J Clin Microbiol* **37**, 2943-51.

Mellors, J. W., Bazmi, H. Z., Schinazi, R. F., Roy, B. M., Hsiou, Y., Arnold, E., Weir, J., and Mayers, D. L. (1995a): Novel mutations in reverse transcriptase of human immunodeficiency virus type 1 reduce susceptibility to foscarnet in laboratory and clinical isolates. *Antimicrob Agents Chemother* **39**, 1087-92.

Mellors, J. W., Kingsley, L. A., Rinaldo, C. R., Jr., Todd, J. A., Hoo, B. S., Kokka, R. P., and Gupta, P. (1995b): Quantitation of HIV-1 RNA in plasma predicts outcome after seroconversion. *Ann Intern Med* **122**, 573-9.

Mellors, J. W., Munoz, A., Giorgi, J. V., Margolick, J. B., Tassoni, C. J., Gupta, P., Kingsley, L. A., Todd, J. A., Saah, A. J., Detels, R., Phair, J. P., and Rinaldo, C. R., Jr. (1997): Plasma viral load and CD4+ lymphocytes as prognostic markers of HIV-1 infection. *Ann Intern Med* **126**, 946-54.

Menendez-Arias, L. (2002): Targeting HIV: antiretroviral therapy and development of drug resistance. *Trends Pharmacol Sci* **23**, 381-8.

Meyer, P. R., Matsuura, S. E., Mian, A. M., So, A. G., and Scott, W. A. (1999): A mechanism of AZT resistance: an increase in nucleotide-dependent primer unblocking by mutant HIV-1 reverse transcriptase. *Mol Cell* **4**, 35-43.

Meyer, P. R., Matsuura, S. E., Schinazi, R. F., So, A. G., and Scott, W. A. (2000): Differential removal of thymidine nucleotide analogues from blocked DNA chains by human immunodeficiency virus reverse transcriptase in the presence of physiological concentrations of 2'-deoxynucleoside triphosphates. *Antimicrob Agents Chemother* **44**, 3465-72.

Meyer, P. R., Matsuura, S. E., So, A. G., and Scott, W. A. (1998): Unblocking of chain-terminated primer by HIV-1 reverse transcriptase through a nucleotide-dependent mechanism. *Proc Natl Acad Sci U S A* **95**, 13471-6.

Milavetz, B. I., Horoszewicz, J. S., Evans, M. J., Manly, K. F., Rinehart, K. L., Jr., and Carter, W. A. (1977): Reverse transcription, a probe by the immobilized template poly(adenylic acid)-agarose. *Mol Pharmacol* **13**, 496-503.

Milavetz, B. I., Horoszewicz, J. S., Rinehart, K. L., Jr., and Carter, W. A. (1978): Study of ansamycin inhibition of a ribonucleic acid-directed deoxyribonucleic acid polymerase by an immobilized template assay. *Antimicrob Agents Chemother* **13**, 435-40.

Miller, M. D., Margot, N. A., Hertogs, K., Larder, B., and Miller, V. (2001): Antiviral activity of tenofovir (PMPA) against nucleoside-resistant clinical HIV samples. *Nucleosides Nucleotides Nucleic Acids* **20**, 1025-8.

Mocroft, A., Vella, S., Benfield, T. L., Chiesi, A., Miller, V., Gargalianos, P., d'Arminio Monforte, A., Yust, I., Bruun, J. N., Phillips, A. N., and Lundgren, J. D. (1998):

Changing patterns of mortality across Europe in patients infected with HIV-1. EuroSIDA Study Group. *Lancet* **352**, 1725-30.
Molla, A., Granneman, G. R., Sun, E., and Kempf, D. J. (1998): Recent developments in HIV protease inhibitor therapy. *Antiviral Res* **39**, 1-23.
Moss, A. R., and Bacchetti, P. (1989): Natural history of HIV infection. *Aids* **3**, 55-61.
Myers, G. (1994): Tenth anniversary perspectives on AIDS. HIV: between past and future. *AIDS Res Hum Retroviruses* **10**, 1317-24.
Nadal, D., Boni, J., Kind, C., Varnier, O. E., Steiner, F., Tomasik, Z., and Schupbach, J. (1999): Prospective evaluation of amplification-boosted ELISA for heat-denatured p24 antigen for diagnosis and monitoring of pediatric human immunodeficiency virus type 1 infection. *J Infect Dis* **180**, 1089-95.
Nibbs, R. J., Yang, J., Landau, N. R., Mao, J. H., and Graham, G. J. (1999): LD78beta, a non-allelic variant of human MIP-1alpha (LD78alpha), has enhanced receptor interactions and potent HIV suppressive activity. *J Biol Chem* **274**, 17478-83.
Nkengasong, J. N., Peeters, M., vanden Haesevelde, M., Musi, S. S., Willems, B., Ndumbe, P. M., Delaporte, E., Perret, J. L., Piot, P., and van den Groen, G. (1993): Antigenic evidence of the presence of the aberrant HIV-1ant70 virus in Cameroon and Gabon. *Aids* **7**, 1536-8.
Novitsky, V. A., Montano, M. A., McLane, M. F., Renjifo, B., Vannberg, F., Foley, B. T., Ndung'u, T. P., Rahman, M., Makhema, M. J., Marlink, R., and Essex, M. (1999): Molecular cloning and phylogenetic analysis of human immunodeficiency virus type 1 subtype C: a set of 23 full-length clones from Botswana. *J Virol* **73**, 4427-32.
Palella, F. J., Jr., Delaney, K. M., Moorman, A. C., Loveless, M. O., Fuhrer, J., Satten, G. A., Aschman, D. J., and Holmberg, S. D. (1998): Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. *N Engl J Med* **338**, 853-60.
Pantaleo, G., Graziosi, C., and Fauci, A. S. (1993): New concepts in the immunopathogenesis of human immunodeficiency virus infection. *N Engl J Med* **328**, 327-35.
Petropoulos, C. J., Parkin, N. T., Limoli, K. L., Lie, Y. S., Wrin, T., Huang, W., Tian, H., Smith, D., Winslow, G. A., Capon, D. J., and Whitcomb, J. M. (2000): A novel phenotypic drug susceptibility assay for human immunodeficiency virus type 1. *Antimicrob Agents Chemother* **44**, 920-8.
Ren, J., Esnouf, R., Hopkins, A., Ross, C., Jones, Y., Stammers, D., and Stuart, D. (1995): The structure of HIV-1 reverse transcriptase complexed with 9-chloro-TIBO: lessons for inhibitor design. *Structure* **3**, 915-26.
Rodgers, D. W., Gamblin, S. J., Harris, B. A., Ray, S., Culp, J. S., Hellmig, B., Woolf, D. J., Debouck, C., and Harrison, S. C. (1995): The structure of unliganded reverse transcriptase from the human immunodeficiency virus type 1. *Proc Natl Acad Sci U S A* **92**, 1222-6.
Sakaguchi, K., Zambrano, N., Baldwin, E. T., Shapiro, B. A., Erickson, J. W., Omichinski, J. G., Clore, G. M., Gronenborn, A. M., and Appella, E. (1993): Identification of a binding site for the human immunodeficiency virus type 1 nucleocapsid protein. *Proc Natl Acad Sci U S A* **90**, 5219-23.
Sandström, E., and Öberg, B. (1993): Antiviral therapy in human immunodeficiency virus infections. Current status (Part I). *Drugs* **45**, 488-508.
Sarafianos, S. G., Das, K., Clark, A. D., Jr., Ding, J., Boyer, P. L., Hughes, S. H., and Arnold, E. (1999a): Lamivudine (3TC) resistance in HIV-1 reverse transcriptase involves steric hindrance with beta-branched amino acids. *Proc Natl Acad Sci U S A* **96**, 10027-32.

Sarafianos, S. G., Das, K., Ding, J., Boyer, P. L., Hughes, S. H., and Arnold, E. (1999b): Touching the heart of HIV-1 drug resistance: the fingers close down on the dNTP at the polymerase active site. *Chem Biol* **6**, R137-46.

Schols, D., Este, J. A., Henson, G., and De Clercq, E. (1997): Bicyclams, a class of potent anti-HIV agents, are targeted at the HIV coreceptor fusin/CXCR-4. *Antiviral Res* **35**, 147-56.

Schuurman, R., Demeter, L., Reichelderfer, P., Tijnagel, J., de Groot, T., and Boucher, C. (1999): Worldwide evaluation of DNA sequencing approaches for identification of drug resistance mutations in the human immunodeficiency virus type 1 reverse transcriptase. *J Clin Microbiol* **37**, 2291-6.

Seage, G. R., 3rd, Oddleifson, S., Carr, E., Shea, B., Makarewicz-Robert, L., van Beuzekom, M., and De Maria, A. (1993): Survival with AIDS in Massachusetts, 1979 to 1989. *Am J Public Health* **83**, 72-8.

Shi, C., and Mellors, J. W. (1997): A recombinant retroviral system for rapid in vivo analysis of human immunodeficiency virus type 1 susceptibility to reverse transcriptase inhibitors. *Antimicrob Agents Chemother* **41**, 2781-5.

Shih, C. K., Rose, J. M., Hansen, G. L., Wu, J. C., Bacolla, A., and Griffin, J. A. (1991): Chimeric human immunodeficiency virus type 1/type 2 reverse transcriptases display reversed sensitivity to nonnucleoside analog inhibitors. *Proc Natl Acad Sci U S A* **88**, 9878-82.

Shirasaka, T., Kavlick, M. F., Ueno, T., Gao, W. Y., Kojima, E., Alcaide, M. L., Chokekijchai, S., Roy, B. M., Arnold, E., Yarchoan, R., and Mitsuya, H. (1995): Emergence of human immunodeficiency virus type 1 variants with resistance to multiple dideoxynucleosides in patients receiving therapy with dideoxynucleosides. *Proc Natl Acad Sci U S A* **92**, 2398-402.

Simon, F., Mauclere, P., Roques, P., Loussert-Ajaka, I., Muller-Trutwin, M. C., Saragosti, S., Georges-Courbot, M. C., Barre-Sinoussi, F., and Brun-Vezinet, F. (1998): Identification of a new human immunodeficiency virus type 1 distinct from group M and group O. *Nat Med* **4**, 1032-7.

Spence, R. A., Kati, W. M., Anderson, K. S., and Johnson, K. A. (1995): Mechanism of inhibition of HIV-1 reverse transcriptase by nonnucleoside inhibitors. *Science* **267**, 988-93.

St Clair, M. H., Martin, J. L., Tudor-Williams, G., Bach, M. C., Vavro, C. L., King, D. M., Kellam, P., Kemp, S. D., and Larder, B. A. (1991): Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase. *Science* **253**, 1557-9.

Stuyver, L., Wyseur, A., Rombout, A., Louwagie, J., Scarcez, T., Verhofstede, C., Rimland, D., Schinazi, R. F., and Rossau, R. (1997): Line probe assay for rapid detection of drug-selected mutations in the human immunodeficiency virus type 1 reverse transcriptase gene. *Antimicrob Agents Chemother* **41**, 284-91.

Subbarao, S., and Schochetman, G. (1996): Genetic variability of HIV-1. *Aids* **10 Suppl A**, S13-23.

Tachedjian, G., French, M., and Mills, J. (1998): Coresistance to zidovudine and foscarnet is associated with multiple mutations in the human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob Agents Chemother* **42**, 3038-43.

Tachedjian, G., Mellors, J., Bazmi, H., Birch, C., and Mills, J. (1996): Zidovudine resistance is suppressed by mutations conferring resistance of human immunodeficiency virus type 1 to foscarnet. *J Virol* **70**, 7171-81.

Tindall, B., and Cooper, D. A. (1991): Primary HIV infection: host responses and intervention strategies. *Aids* **5**, 1-14.

Trono, D. (1995): HIV accessory proteins: leading roles for the supporting cast. *Cell* **82**, 189-92.

UNAIDS (2002): AIDS epidemic undate: December 2002. UNAIDS, Geneva, Switzerland.

Vaishnav, Y. N., and Wong-Staal, F. (1991): The biochemistry of AIDS. *Annu Rev Biochem* **60**, 577-630.

Van Laethem, K., Witvrouw, M., Balzarini, J., Schmit, J. C., Sprecher, S., Hermans, P., Leal, M., Harrer, T., Ruiz, L., Clotet, B., Van Ranst, M., Desmyter, J., De Clercq, E., and Vandamme, A. M. (2000): Patient HIV-1 strains carrying the multiple nucleoside resistance mutations are cross-resistant to abacavir. *Aids* **14**, 469-71.

Vandamme, A. M., Van Vaerenbergh, K., and De Clercq, E. (1998): Anti-human immunodeficiency virus drug combination strategies. *Antivir Chem Chemother* **9**, 187-203.

Vanden Haesevelde, M., Decourt, J. L., De Leys, R. J., Vanderborght, B., van der Groen, G., van Heuverswijn, H., and Saman, E. (1994): Genomic cloning and complete sequence analysis of a highly divergent African human immunodeficiency virus isolate. *J Virol* **68**, 1586-96.

Verma, I. M. (1977): The reverse transcriptase. *Biochim Biophys Acta* **473**, 1-38.

Wainberg, M. A., and White, A. J. (2001): Current insights into reverse transcriptase inhibitor-associated resistance. *Antivir Ther* **6 Suppl 2**, 11-9.

Wei, X., Ghosh, S. K., Taylor, M. E., Johnson, V. A., Emini, E. A., Deutsch, P., Lifson, J. D., Bonhoeffer, S., Nowak, M. A., Hahn, B. H., Saag, M. S., and Shaw, G. M. (1995): Viral dynamics in human immunodeficiency virus type 1 infection. *Nature* **373**, 117-22.

Whiting, S. H., and Champoux, J. J. (1994): Strand displacement synthesis capability of Moloney murine leukemia virus reverse transcriptase. *J Virol* **68**, 4747-58.

Winters, M. A., Coolley, K. L., Girard, Y. A., Levee, D. J., Hamdan, H., Shafer, R. W., Katzenstein, D. A., and Merigan, T. C. (1998): A 6-basepair insert in the reverse transcriptase gene of human immunodeficiency virus type 1 confers resistance to multiple nucleoside inhibitors. *J Clin Invest* **102**, 1769-75.

Yamamoto, S., Folks, T. M., and Heneine, W. (1996): Highly sensitive qualitative and quantitative detection of reverse transcriptase activity: optimization, validation, and comparative analysis with other detection systems. *J Virol Methods* **61**, 135-43.

Yang, G., Song, Q., Charles, M., Drosopoulos, W. C., Arnold, E., and Prasad, V. R. (1996): Use of chimeric human immunodeficiency virus types 1 and 2 reverse transcriptases for structure-function analysis and for mapping susceptibility to nonnucleoside inhibitors. *J Acquir Immune Defic Syndr Hum Retrovirol* **11**, 326-33.

Yu, H., and Goodman, M. F. (1992): Comparison of HIV-1 and avian myeloblastosis virus reverse transcriptase fidelity on RNA and DNA templates. *J Biol Chem* **267**, 10888-96.

Zhou, W., and Resh, M. D. (1996): Differential membrane binding of the human immunodeficiency virus type 1 matrix protein. *J Virol* **70**, 8540-8.