CHAPTER II

LITERATURE REVIEW

Since the acquired immunodeficiency syndrome (AIDS) was first recognized in the United States in 1981, the number of cases has risen swiftly worldwide. Knowledge of AIDS has also increased proportionately. Barre-Sinoussi F, Chermann JC, and Montagnier L at the Pasteur Institutes in Paris and a group led by Gallo RC at the National Institute of Health, USA, independently identified the causative agent: a virus of the retrovirus family, in 1993 and 1984, respectively.[21] The French group called it LAV (Lymphadenopathy-associated virus) and the American named: HTLV-III (human T-lymphotropic virus type III) and AIDS-related retrovirus (ARV).[22,23] Further studies of the virus, it was found that LAV similar to HTLV-III in infecting CD4 T-lymphocytes, but had different properties. In 1986, however, an expert committee, empowered by the International Committee on the Taxonomy of Virus, agreed on the name human immunodeficiency virus (HIV) and this name has been used ever since.[24,25]

Now, the HIV can be further classified into HIV-1 (the original virus) and HIV-2 (the newly discovered variant). Both types of HIV infect patient's immune system, but the overall nucleotide sequence homology between HIV-1 and HIV-2 is only 42 percent.[26] The HIV-1 is found primarily in Central Africa, Europe and the United States whereas HIV-2 is mainly found in Western Africa.[27] HIV-2 also causes AIDS but possible with a slower evolution than that of HIV-1.[24,28]

ANTIRETROVIRAL THERAPY

During the past 10 years significant progress has been made in the treatment of HIV-infected patients, in part due to the development and clinical use of an increasing number of anti-HIV drugs. The Food and Drug Administration (FDA) have approved three classes of drugs. They are targeted at only 2 events in the HIV replication cycles: (i) reverse transcription of the viral RNA into double-stranded proviral DNA by the viral reverse transcriptase (RT), and (ii) processing of the viral precursor gag-pol protein by the viral protease (PR). Both the RT and PR are virus-

specific enzymes and essential for replication. They are therefore excellent targets for antiviral therapy.

Two classes of RT inhibitors are being successfully used at present: nucleoside analogue RT inhibitors (NRTIs), acting as competitive inhibitors and chain terminators, and non-nucleoside RT inhibitors (NNRTIs), exerting an allosteric effect by binding to a hydrophobic pocket close to the active site.[29] The PR inhibitors (PIs) in current use are targeted at the active site of the enzyme.[30] The most recent approval RT inhibitor is a nucleotide RTI : tenofovir.[1]

NUCLEOSIDE ANALOGUES REVERSE TRANSCRIPTASE INHIBITORS (NRTIs)

NRTIs comprise the first class of drug with proven antiretroviral efficacy against HIV-1. These drugs act by competitively inhibiting HIV reverse transcriptase activity. After entry into target cells, these drugs are converted into the active triphosphorylated form by cellular enzyme. Viral reverse transcriptase may be inhibited by these compounds or may take up the compounds and insert them in the growing DNA proviral chain. Chain termination results because subsequent nucleosides do not have the deoxyribosylhydroxyl group for attachment of the next link. Six nucleoside analogues are currently available: zidovudine (AZT, ZDV), didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC) and abacavir (ABC). All NRTIs are safe, generally well tolerated, and effective in prolonging life, particularly when used in combination regimens with other antiretroviral drugs. They also delay or prevent the development of HIV resistance to protease inhibitors and non-nucleoside reverse transcriptase inhibitors.

Zidovudine (Azidothymidine : AZT)

The dideoxynucleoside; 3'-azido-2'-3'-dideoxythymidine (Zidovudine) was the first antiretroviral drug approved for use in treatment of AIDS in 1985.[31]

Zidovudine is a thymidine analogue in which the 3'-hydroxy (-OH) group is replaced by an azido (-N3) group. It inhibits proviral DNA synthesis in two ways: competitive inhibition and chain termination. Registration for this indication was based on trials which demonstrated that zidovudine had antiretroviral activity and that

it delayed HIV progression and improved survival in HIV-1-infected patients with advanced disease.[31,32,33]

Didanosine (2',3'-dideoxyinosine : ddI)

Didanosine was licensed in 1992. It became the second antiretroviral agent to receive regulatory approved in the U.S. and Canada. On the strength of trial ACTG 116B/117, which studied moderate to severe HIV illness in persons who received zidovudine for 16 or more weeks. This study demonstrated that in asymptomatic patients and those with AIDS-related complex, didanosine significantly delayed the time to the first AIDS-defining event or death, compared to zidovudine.[34,35]

Zalcitabine (2',3'-dideoxycytidine : ddC)

In 1992, zalcitabline was approved in combination with AZT for treatment of AIDS. Zalcitabine has shown additive or synergistic effect against HIV. It can inhibit zidovudine resistant HIV isolated and can improve the CD4+ cell counts. However, with zalcitabine, peripheral neuropathy is the side effect which most often limits dosage in zalcitabine monotherapy.[36] Clinical data supporting the long-term efficacy are perhaps more limited with zalcitabine than any of the other nucleoside analogues.

Stavudine (2',3'-Didehydro-2',3'-dideoxythymidine : d4T)

Stavudine was approved by the FDA in 1993 for the treatment of HIV-infected adults who have received prolonged zidovudine therapy.[37] In phase I trials, it increases in CD4+ cell counts and suppression of p24 antigenemia. Toxicities include neuropathy, elevations of liver transaminases, and anemia.[38]

Lamivudine (2'-deoxy-3'-thiacytidine : 3TC)

In 1995, lamivudine was approved for HIV therapy with a good oral bioavailability and less toxicity than zidovudine. Lamivudine has activity against a wide spectrum of HIV-1 isolates, including those that are zidovudine resistant.[39] Rapid selection of resistant virus has been observed with lamivudine *in vitro* [39,40] and in vivo.[41] Although the rapid emergence of codon 184 mutant lamivudine-resistant virus limits its use as a single agent, the same mutation at codon 184 suppresses

zidovudine resistance,[39] which suggests a potential mechanism for the sustained activity of the combination of zidovudine and lamivudine.[42]

Abacavir (ABC)

Abacavir is an analogue of the nucleoside guanine. ABC is metabolized intracellularly to a related nucleoside analogue (carbovir). The FDA approved ABC in 1998.

NUCLEOTIDE ANALOGUE REVERSE TRANSCRIPTASE INHIBITORS

Nucleotide analogue is very similar to nucleotide analogues. The only difference is that nucleotide analogue is chemically preactivated and thus required less biochemical processing in the body for them to become active.

Tenofovir (TDF)

Tenofovir is now approved for the treatment HIV by the US-FDA in 2001. Recent study indicates that TDF may reduce levels of HIV RNA circulating in the bloodstream by more than 80%, and work effectively in patients who have displayed resistance to a wild variety of other antiretroviral drugs, particularly nucleoside analogs.[1] This highly active compound also appears to cause very few side effects.

PROTEASE INHIBITORS (PIs)

The HIV-1 protease enzyme is an important target for the design of anti-HIV drug. Because mutations on the active site of the protease molecule have been found to prevent complete processing of the virus. From this and other studies, viral load reduction in response to treatment is now regarded as an important tool in monitoring antiretroviral therapy.[43]

When initially introduced, protease inhibitors resulted in widespread optimism among clinicians and patients that HIV may become a long-term, manageable disease. It reduce HIV load by up to 99% and increase CD4+ T cell counts by over 100 cells/mm^3 of blood. To achieve long-term viral suppression, protease inhibitor therapy must be managed carefully.[44,45] The six protease inhibitors currently licensed by the

FDA for use in the United States are saquinavir (SQV), ritonavir (RTV), indinavir (IDV), nelfinavir (NFV), amprenavir (APV), and lopinavir (LPV).

NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NNRTIs)

In 1996, a subcommittee at the FDA recommended accelerated approval for nevirapine, thereby introducing a third class of anti-HIV therapies: the non-nucleoside reverse transcriptase inhibitors (NNRTIs). As a class, the NNRTIs are heterogeneous with respect to chemical structure. NNRTIs bind noncompetitively to the HIV reverse transcriptase enzyme, causing a disruption in the enzyme's catalytic site.[46] In contrast to the nucleoside analogues, NNRTIs do not require intracellular phosphorylation to become active. Although the two antiretroviral classes inhibit the same enzyme, there is no evidence for cross-resistance between the two classes. There are currently three NNRTIs available worldwide: navirapine (NVP), delavirdine (DLV), and efavirenz (EFV)

Nevirapine (NVP)

Nevirapine was the first available compound in NNRTIs class. The development of navirapine was delayed due to the observation that high level resistance emerges rapidly when the drug is used as monotherapy.[47]

Delavirdine (DLV)

In 1997, the FDA approved delavirdine for use in the treatment of HIV infection. Delavirdine is chemically very different from navirapine. Like all NNRTIs, the mechanism of action of NNRTIs is very different from nucleoside analogues (such as zidovudine) Therefore delavirdine is often used in combination with these agents. Delavirdine does not have activity against HIV-2

Efavirenz (EFV)

Efavirenz was recently approved for the treatment of HIV infection. Although efavirenz has the same mechanism of action as navirapine and delavirdine, it has several unique properties that make it a very effective and popular anti-HIV therapeutic.

COMBINATION THERAPY

Combination therapy for controlling HIV-1 infection provides several potential advantages for instance higher overall efficacy, lower toxicity and possibly diminish the risk of drug resistance. Several drug combinations have been shown to act synergistically against HIV in vitro. Not all drug combinations will necessarily lead to synergism, however, and some may simply have an additive effect whilst other may have an antagonistic effect. Combination therapy could target different cellular and tissue reservoirs of virus. There is also a rational basis for combining antiviral drugs that act at different stages of the HIV replication cycle.[48] When combined, such drugs may provide a double barrier; prevent shedding of the virus from its reservoirs and prevent spread of the virus and recruit of new cells, so keeping the HIV infection under complete control.

Accumulating data from clinical and pathogenesis studies continue to support combination therapy in patients with HIV infection. A variety of combination regimens show potency, expanding choices of regimens for individual patients, for instance, two NRTIs (the first combination regimen), three NRTIs, two NRTIs plus NNRTI, two PIs, or 2 NRTIs plus PI (highly active antiretroviral therapy, HAART).

ANTIRETROVIRALS AVAILABLE IN THAILAND

Currently there are a total of 13 antiretroviral agents available in Thailand (Tenofovir, delaverdine and amprenavir are not licensed). To bring the cost of treatment down, the Government Pharmaceutical Organization (GPO) are manufacturing generic 4 NRTIs (AZT, d4T, ddI, and 3TC) and one generic NNRTI (nevirapine). The fixed dose combination formula generic of AZT/3TC and d4T/3TC/nevirapine are also available. The cost triple therapy in Thailand at present has been markedly reduced to approximately 1,200 Bath or 30 US$ per month. This certainly has made antiretroviral therapy being more accessible to patients needed.

GOLDS OF ANTIRETROVIRAL THERAPY

Eradication of HIV infection cannot be achieved with currently available antiretroviral regimens; in large measure, this is due to the establishment of a pool of infected CD4+ T cells during the very earliest stages of acute HIV infection [49] that persists with an extremely long half-life, even with prolonged suppression of plasma viremia to < 50 copies/mL.[50,51,52,53] The primary goals of antiretroviral therapy are maximal and durable suppression of viral load, restoration and/or preservation of immunologic function, improvement of quality of life, and reduction of HIV-related morbidity and mortality. In fact, adoption of treatment strategies articulated in these guidelines has results in substantial reductions in HIV-1 related morbidity and mortality.[54,55]

The plasma HIV RNA levels (viral load) and CD4+ T cell count testing should guide decision regarding initiation or changes in antiretroviral therapy, as well as the clinical condition of the patient. The results of these two tests give the clinician important information about the virologic and immunologic status of the patients and the risk of disease progression to AIDS.[56,57]

Plasma viremia is a strong prognostic indicator in HIV infection.[56] Furthermore, reductions in plasma viremia achieved with antiretroviral therapy account for much of the clinical benefit associated with therapy.[58] Therefore, suppression of plasma viremia as much as possible for as long as possible is an important goal of antiretroviral therapy. However, this goal must be balanced against the need to preserve effective treatment options. Switching antiretroviral regimens for any detectable level of plasma viremia may rapidly exhaust treatment options.

Highly active antiretroviral therapy (HAART) often leads to increases in the CD4+ T cell count of 100-200 cells/μL or more, although individual responses are quite variable. CD4+ T cell response are generally related to the degree of viral load suppression.[59] In turn, continued viral load suppression is more likely among those who achieve higher CD4+ T cell counts.[60] A favorable CD4+ T cell response can occur with incomplete viral load suppression and may not necessary indicate a poor prognosis.[61] The durability of these immunologic responses that occur with suboptimal suppression of viremia is unknown. Therefore, while viral load is the strongest single predictor of long-term clinical outcomes, strong consideration should

also be given to sustained rises in CD4+ T cell counts and partial immune restoration. The urgency of the need to change therapy in the presence of low level viremia is clearly tempered by this observation. The expectation that continuing the existing therapy in this situation will inevitably lead to rapid accumulation of drug resistant virus may not always be realized. One reasonable strategy is maintenance of the regimen, but with redoubled efforts at optimizing adherence, and more frequent monitoring.

MECHANISMS OF RESISTANCE

Despite the success of antiretroviral therapy (ART) in reducing HIV progression, initial and subsequent regimens fail in many patients. Cohort studies have shown that in approximately 50% of patients, HIV RNA rebound above detection developed or initial suppression could not be achieve.[62] Data form one primary care clinic suggest that the proportion of patients who received all three classes of ART is increasing. These data point to a growing number of patients whose ART regimens have failed and who require changes in therapy. Although there are many possible causes for regimen failure, such as poor adherence, lack of regimen potency, and inadequate drug concentrations, resistance to antiretroviral agents remains an important cause and consequent of treatment failure.

HIV drug resistance is a result of the high replication and mutation rates of HIV combined with the capacity of HIV for genomic integration and dormancy.[3] The half-life of plasma virions can be as short as 6 hours, with 10 billion virions produced each day in the untreated patient. HIV reverse transcriptase (RT), encoded by the polymerase, or pol, gene, lacks a 3' to 5' exonuclease activity, or "proofreading" ability, thereby enables spontaneous mutations to occur regularly.[63,64] An average of 1 mutation occurs during each replication cycle, thus virtually every single drug-resistance mutation to occur at least once a day. Most of these mutations are detrimental to viral replication, but some may retain enough replicative capacity (or "fitness") to persist in circulation.[2,4,5] This high mutation rate generates a complex of genetically related but distinguishable variants called quasispecies.[4]

In the presence of environmental pressures created by drug therapy, only the "fittest" viral strains will propagate and emerge as dominant strains. Changing drug

therapies will select for a different set of mutant strains. Even if a drug-resistant strain accounts for only a small minority of the overall viral population, selective pressures from antiretroviral therapy will favor the replication and propagation of that resistant strain.[65]

Resistance to antiretroviral drugs is determined by mutations in the genes that encode the PR and RT. Primary mutations cause decreased binding of the drug to its enzyme target and are the first mutations selected during therapy. This results in an increased amount of drug required to inhibit the enzyme. Secondary mutations contribute to drug resistance by improving the fitness of viruses carrying primary mutations. They have little direct effect on inhibitor binding or on the level of resistance in the absence of primary mutations.

NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITOR RESISTANCE

Resistance to NRTIs results from base changes within the RT genome, allowing amino acid substitutions in the RT enzyme, which in turn confer structural changes at the enzyme active site or associated functional areas. (Table I)

Table I : Mutations in *RT* gene associated with reduced susceptibility to NRTIs [12,66]

Mutations / NRTIs	E	M	A	K	D	T	K	L	V	F	F	V+	M	Q	L	T	K
	44	41	62	65	67	69	70	74	75	77	116	118	184	151	210	215	219
	D	L	V	R	N	S,D	R	V	I	L	Y	I	V	M	W	Y/F	Q/E
Multi-nRTI Resistance																	
- 151 Complex			+						+	+	+			+			
- 69 insertion		+	+		+	+[a]	+								+	+	+
- NAMs		*			*		*								*	*	*
Zidovudine		+			+		+								+	+	+
Didanosine		*		+	*		*	+					+		*	*	*
Zalcitabine		*		+	*	+[b]	*	+					+		*	*	*
Stavudine		+			+		+		+[c]						+	+	+
Abacavir		+		+	+		+	+					+		+	+	+
Lamivudine	+											+	+				

[a] insertion by amino acid S; [b] insertion by amino acid D; [c] selected in Vitro

+ indicate mutation; * and vertical lines indicate NAMs

Zidovudine Resistance

The development of HIV resistance to AZT, first described in 1989, has been well characterized.[67,68] The incidence of AZT resistance is related to several factors, including duration of treatment and disease stage. In early studies of AZT monotherapy, the emergence of phenotypically resistant strains correlated with the duration of AZT monotherapy.[69,70]

Resistance also appears to develop more rapidly in patients with advanced disease and high levels of viral replication.[70,71] These observations likely apply to all antiretroviral therapies. Genotypic analysis of AZT-resistant strains demonstrated that for the development of high-level resistance, five mutations in the RT gene are critical. These mutations occur at codons 70, 215, 41, 67, and/or 219.[10,68] These mutations typically evolve in a sequential manner.[10]

The K70R mutation usually is the first mutational change in HIV-1 RT to emerge during AZT therapy.[10] Although this mutation produces only a modest (8-fold) decrease in AZT susceptibility in molecular clones of HIV-1,[20] variants of HIV-1 carrying the K70R mutation are selected rapidly *in vivo* after initiation of AZT therapy.[72] Subsequently, variants with the T215Y/F and M41L mutations emerge and replace the K70R mutants. The combined presence of mutations at these two codons confers a 60-fold increase in IC_{50}.[10,73] Continued evolution leads to re-emergence of the K70R mutation as well as accumulation of the D67N and K219Q mutations. The combined presence of all five mutations results in a 500- to 1000-fold increase in IC_{50} as compared to wild-type virus. Recently, a sixth mutation (L210W) has been identified that enhances slightly the level of AZT resistance and associated with long-term AZT treatment.[74] High level AZT resistance is associated with broad cross-resistance to other nucleoside analogues.

Of note, recent studies have found that these originally named as AZT-resistant mutations, in fact can be indused by other NRTIs : d4T, ddI, ddC, ABC. Thus they were currently called **nucleoside analog mutations or NAMs**. Furthermore, 4 NAMs or more mutation shown to cross-resistant within the NRTI class except lamivudine.[12]

Didanosine Resistance

Resistance viruses may emerge after months of ddI therapy. The L74V,[75,76] M184V,[77] and K65R[78] mutations correlate with decreased HIV sensitivity to ddI. The L74V mutation appears to be most common and confers cross-resistance to zalcitabine; however, it was the first of several mutations that have been shown to reverse AZT resistance.[76] Emergence of the codon 74 mutation is prevented or delayed in patients treated with ddI in combination with AZT.[15] Conversely, emergence of AZT resistance was not delayed by the combination.[15,79,80] Didanosine rarely can select for the M184V mutation, which also confers resistance to 3TC.

Zalcitabine Resistance

Diminished susceptibility to zalcitabine is not well described. Genotypic patterns from patients receiving long-term ddC suggest that K65R, T69D, L74V, and M184V/I mutations are associated with resistance to the drug. Cross-resistance to ddI has been seen with the K65R but not with the T69D mutations.[80]

Stavudine Resistance

There is even less experience with resistance to stavudine. The V75T mutation may confer on HIV reduced susceptibility to the drug. The clinical relevance of these mutations is unknown. Recent data indicate that long-term exposure to d4T may select for AZT related mutations [81] and currently known as **thymidine analog resistance mutations (TAMs)**.[11]

Lamivudine Resistance

Resistance to lamivudine occurs rapidly in vitro and in vivo, and is associated with a mutation at codon 184. M184V (sometimes preceded by an "M184I" mutation) [42] occurs very rapidly after exposure to lamivudine (unless complete viral suppression is achieved) [41] and confers up to a 1,000-fold decrease in sensitivity to the drug.[40,41] Because the M184V mutation can be selected by ddI or ddC, there has been concern regarding the extent of cross-resistance between these drugs and 3TC. [82] M184V delays the development of AZT resistance and may restore AZT activity

once resistance emerges.[40] This observation, confirmed in several clinical trials, supports the widespread use of AZT and 3TC in combination.[75]

Multi-Nucleoside Resistance (MNR) : the Q151M, T69S insertion complex

Unique mutations in the gene for reverse transcriptase can occur in patients receiving combination nucleoside analogue therapy. The Q151M substitution appears first and is thought to be crucial for subsequent development of multiple nucleoside analog resistance.[13] This mutation results in a high level cross-resistance to all nucleoside analogues when associated with mutations at codons 62, 75, 77, and 116. [14,82,83] This complex of mutations is associated with long-term exposure to AZT and ddI.

Multinucleoside resistance can be seen along a second pathway: the so-called T69S insertion mutation. Up to six nucleotides may be inserted at or near codon 69 (resulting in two additional serine residues). This mutation confers high-level cross resistance to all nucleoside analogues (particularly when combined with classic AZT related mutations, particularly T215Y). It appears to be relatively uncommon, and occurs in patients who have long-term exposure to nucleoside analogue therapy (particularly ddI or ddC in combination with AZT).[84]

Finally, the combination of mutations selected by long term exposure to AZT (mutations M41L, T215Y/F, 67N, K70R, L215W, K219E/Q) may be a third form of multi-nucleoside resistance.

NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITOR RESISTANCE

Although the various NNRTIs are chemically diverse, they act in the same hydrophobic binding site within RT. It is therefore not surprising that developing resistance to one NNRTI often confers significant cross-resistance to most other NNRTIs. NNRTI resistance mutations occur in two regions of RT gene: codons 98 to 108 and codons 179 to 190. None of the mutations overlaps with mutations that cause resistance to NRTIs. (Table II)

Table II : Most common mutations in HIV selected by NNRTIs [12,85]

Multi-NNRTI resistance		**K103N**				**Y188L**			
Multi-NNRTI (accumulating of mutations)	L100I		V106A		**Y181C,I**		**G190S/A**	M230L	
Nevirapine		**K103N**	**V106A**	**V108I**	**Y181C,I**	**Y188C**	**G190A**		
Delavirdine		**K103N**			**Y181C,I**				P236L
Efavirenz	L100I	**K103N**			Y181C,I		G190E/S		P255H

Mutations **in bold** are primary mutations.

The most common changes involve a K103N mutation (selected by EFV, DLV, and NVP) and a Y181C mutation (selected by DLV and NVP). Mutations at codon 181 directly disrupt binding of NVP to RT; the K103N mutation acts indirectly by inhibiting formation of the drug-binding pocket.[86]

The Y181C mutation is favored by DLV and NVP over K103N, presumably because a single nucleotide change at this position produces 1000-fold resistance.[16,87] By contrast, the level of resistance produced by the K103N mutation is relatively modest (on the order of 20-fold). Additional mutations accumulate following initial emergence of K103N or Y181C, suggesting continued remodeling and adaptation of RT under selective pressure of NNRTI therapy.

Resistance to Nevirapine

High-level resistance to nevirapine occurs rapidly when it is used as monotherapy (in as early as 1 week).[16] Genotypic analysis reveals that mutations in the reverse transcriptase gene at codons 181 and/or 106 predictably confer high-level resistance to nevirapine. Mutations have also been seen at codons 103, 108, 188, and 190. The pattern of resistance changes if NVP is given with nucleoside analogues, particularly zidovudine. The concurrent use of zidovudine appeared to prevent the emergence of the Y181C mutation.[88] Therefore, the emergence of K103N mutation is favored over Y181C.[88,89,90] This observation, since confirmed, is likely to explained by the ability of Y181C to restore sensitivity to zidovudine when zidovudine resistance is already present. The clinical relevance of these observations has not been fully determined. If the Y181C mutation proves to preserve options for

future NNRTI therapy, then strategies based on avoiding zidovudine/nevirapine could be considered.

Resistance to Delavirdine

As with other NNRTIs, resistance to delavirdine develops rapidly when the drug is used as monotherapy,[91] and the K103N and Y181C mutations were common. Passage of HIV-1 in vitro in the presence of delavirdine often selects for a unique mutation at codon 236 (P236L). This mutation confers limited cross-resistance to other NNRTIs, including nevirapine and efavirenz.[92] However, when administered in vivo, the P236L mutation is rarely observed. Cross-resistance after failure of delavirdine will likely be common.

Resistance to Efavirenz

In vitro and *in vivo*, EFV resistance is commonly associated with the K103N mutation. The K103N mutation confers moderate resistance to EFV (approximately 20-fold increase in the IC50) and significant cross-resistance to NVP and DLV. Other mutations may emerge during therapy with EFV (V108I, G190S, P225H, and others). High-level resistance may require multiple mutations within the same genome.

ANTIRETROVIRAL DRUG RESISTANCE TESTING

Use of resistance testing is now recommended to assist in the design of salvage antiretroviral regimens for HIV-1 infected patients.[93] Drug susceptibility of HIV-1 can be measured directly by phenotypic assays or inferred by genotypic assays. Both phenotypic and genotypic resistance assays are becoming more wildly available.

PHENOTYPIC ASSAYS

Phenotypic assays measure the susceptibility of a patient's viral isolate to individual antiretroviral drugs. These assays measure the ability of HIV-1 to grow in the presence of drugs and are performed using assays in which the degree of virus replication inhibition at different drug concentrations is assessed. Results are used to

calculate the 50% or 90% inhibitory concentration (IC_{50} or IC_{90}) of a drug for an isolate. Results also can be presented as *fold-change* in IC_{50} or IC_{90} for each isolate as compared with a drug susceptible control strain or prior isolate from the same patient.

Traditionally, phenotypic resistance of HIV-1 to antiretroviral drugs was measured by culturing patient viruses in HIV negative donor peripheral blood mononuclear cells (PBMCs) and measuring the effects of different concentrations of drug on viral replication in cell culture.[94] This process was not automated and so was time-consuming and expensive. In addition, significant variability in the assay was seen, in part because of the need to use PBMCs from different donors.

The ability to measure phenotypic resistance of HIV-1 isolates on a large scale was made possible with the use of recombinant virus assays.[95,96,97] In this assay method, a region of the viral genome containing the determinants of drug resistance (i.e. PR and RT) is amplified using reverse transcription polymerase chain reaction (RT-PCR) from viral sequences present in patient plasma and then inserted into an HIV vector that is deleted for PR and RT but contains the remainder of the HIV genome. These recombinants are then tested in an automated drug susceptibility assay. Recombinant virus assays reduce the time required for obtaining a result from 6-8 weeks to 2-3 weeks, and substantially reduce variability in the assay. However, these processes are difficult to carry out in local laboratories, and the cost of the tests remains very high.

Two assays that measure HIV-1 phenotypic drug susceptibility are currently available to the clinician: PhenoSense (ViroLogic, U.S.A.) [95] and Antivirogram (Virco, Belgium).[96] Each assay has been extensively validated internally by each company and is performed in a limited number of reference laboratories. However, because these assays differ in various technical aspects and have not been directly compared, their results should not be used interchangeably, and the cost of the tests remains very high.

GENOTYPIC ASSAYS

Whereas phenotypic assay measures virus drug susceptibility, genotypic assay measure the presence of mutations in the RT or PR gene that might confer phenotypic resistance.

All genotypic assays use initial amplification through RT-PCR as the first step in the process. As well as phenotypic assays, genotypic assays usually require samples with more than 1,000 copies/mL of HIV-1 RNA to obtain amplification from plasma, although amplification is possible in some samples at lower viral loads. Inherent in these assay is the concern with false positive, either from carryover from other HIV-1 samples in the laboratory or from random polymerase errors in vitro during in vitro nucleic acid synthesis.

There are two main types of genotypic assays:

1. DNA Sequencing (The standard assay) : This assay determines the entire nucleotide sequence of the RT and PR gene and provides sequencing information about all regions of the gene, including those that are not necessarily known to confer resistance.

Two methods of sequencing the amplified HIV-1 DNA fragment are used : one is based on *in vitro* copying of amplified DNA templates (dideoxynucleotide terminator cycle sequencing), and the other is based on hybridization of the amplified nucleic acid (sequencing by hybridization).

Modified sequencing technologies are available commercially from PE Biosystems ViroSeq (U.S.A.), Visible Genetics TruGene (Canada) and Affymetrix Gene Chip (U.S.A).[98]

2. Point mutation assay : This assay identifies viral gene sequences that are known to confer resistance. There are two point mutation assays:

2.1 **Probe by hybridization based assay** : This assay is based on the reverse hybridization principle and now commercially available as the line probe assay (LiPA).[99] It involves detecting a nonradioactive colorimetric signal from hybridization of HIV-1 PCR product to oligonucleotide probes immobilized in lines

on a paper strip. Data analysis is simple and fast with this assay; however, it is now available only for NRTIs resistance (codons 41, 69, 70, 74, 184, 214 and 215).

2.2 **Selective PCR** : This assay is based on the selective priming of either wide-type (WT) or mutant-type (MT) primers to the sequence at the codon of interest. The result were analyzed on agarose gels and identified by ethidium bromide staining.

Several studies encountered a problem with selective PCR, that is the mispriming of selective primers.[100,101] Other investigators have also noted the problem, particularly if the initial target input is too high. It has been recommended to dilute the primary PCR product in order to minimize mispriming and to resolve apparent mixtures.[101] This procedure led to the possibility of erroneous conclusion. However, mispriming in PCR is well documented. In their initial report of the "amplification refractory mutation system (ARMS)", mispriming found to be a common problem with most primers.[19] In an attempt to make primers more selective, a series of additional mismatches at various sites in the primers were introduced, which however improved only some primers.

ADVANTAGES AND DISADVANTAGES OF RESISTANCE TESTING

Phenotypic and genotypic assays provide complementary information. Both approaches have distinct advantages and disadvantages, but all assays share certain limitations (Table III). For example, both assays are relatively insensitive to the presence of minor variants within the virus quasispecies. Therefore, most assays fail to detect drug-resistant mutants that constitute <20% of the population.[98] In addition, technical limitations in the initial amplification through RT-PCR as the first step make it difficult to obtains reliable results when the plasma HIV-1 RNA level is < 1,000 copies/mL.[102] Because both assays sample the actively replicating pool of virus, they also may fail to detect the presence of resistance against drugs to which the patient was exposed in the distant past. Such resistant variants persist in the population, however, and may lead to rapid failure of a regimen when previously used drugs are "recycled."

Phenotypic assays measure the actual susceptibility of the virus, although specific susceptibility "break points" have not been validated for most drugs. An important advantage of phenotypic assays is the ability to determine the consequences

of mutational interactions, such as the effect of lamivudine resistance on susceptibility to zidovudine. The major disadvantages of phenotypic assays include their cost, relatively slow turn-around time, and limited availability.

By contrast, genotypic assays can be performed relatively rapidly and at lower cost than phenotypic assays. However, the data produced by such assays often require expert interpretation, particularly in the case of automated gene sequencing. Interpretation of the clinical significance of specific mutations may depend on the laboratory performing the assay, leading to inconsistency in reporting and occasional misinterpretation.

Table III : Comparison of Genotypic and Phenotypic HIV Resistance Assays [102]

Relative Advantages	Relative Limitations
Phenotypic Assays	
Direct measure of susceptibility	Restricted availability
More familiar results (e.g. IC_{50} or IC_{90})*	Longer time to results (weeks)
	Technically demanding
	Insensitive for detecting minor species
	Clinically significant cutoff values undefined
Genotypic Assays	
Availability	Indirect measure of susceptibility
Shorter time to results (days)	May not correlate with phenotype
Less technically demanding	Expert interpretation required
Mutations may likely precede phenotypic resistance	Most methods insensitive for detecting minor species

* IC_{50} or IC_{90} indicate concentrations of drug required for inhibition of the replication of microorganisms at the level represented by the subscripted number (eg. IC_{50} inhibits replication by 50% and IC_{90} inhibits replication by 90%)

CLINICAL USES OF DRUG RESISTANCE TESTING

Assays for HIV-1 drug resistance could potentially be useful in guiding antiretroviral therapy in several ways. These include choice of initial treatment regimen, explaining and managing treatment failure, and tracking the prevalence of primary (e.g., transmitted) drug resistance. Recent data suggest that drug resistance mutations are present in HIV-1 from 10% to 15% of newly-infected individuals, [103,104] although the prevalence of resistance in treatment-naive individuals varies by

region. Transmission of multiply resistant HIV-1 also has been documented.[105] Screening for the presence of drug resistance prior to initiating antiretroviral therapy is sensible, particularly in areas of high prevalence.

For patients with drug-resistant HIV-1, results of resistance testing may help guide the choice of salvage therapy. Drug resistance testing is likely to be most useful in patients failing an initial treatment regimen, and in identifying the presence of resistance to drugs in the currently failing regimen. Resistance to drugs with which the patient has been treated in the past may go undetected as a result of rapid shifts in the HIV-1 quasispecies. Thus, resistance testing will be most useful in identifying drugs to be avoided, but the absence of apparent drug resistance is no guarantee of therapeutic success.

Although antiretroviral drug resistance testing may be useful in selecting initial or subsequent potent antiretroviral regimens, the results of these tests must be interpreted carefully and a patient's treatment history and changes in viral load should also be taken into consideration. CD4+ cell count and plasma HIV RNA level are still the essential factors for guiding when to start treatment and when to change therapies.