Combined Effects of Lavendamycin Analogs and AZT on HIV-Reverse Transcriptase

An Honors Thesis (HONRS 499)

By

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Abstract

Lavendamycin is a naturally occurring antibiotic isolated from *Streptomyces lavendulae*. Like its related antibiotic, streptonigrin, lavendamycin has anti-tumor, anti-viral and anti-bacterial activity and is highly toxic in its natural form. Several recently synthesized lavendamycin analogs were found to have low toxicity and significant activity against HIV-reverse transcriptase. Initial studies have shown at least a few of the novel analogs have considerable anti-HIV-RT activity alone and also synergistic effects when used in combination with AZT.

In this study, four lavendamycin analogs were assessed in vitro for their optimum HIV-RT inhibitory ratios and concentrations with AZT. While most of the analogs showed additive and synergistic activity with AZT, MB 72 and MB 344 exhibited significant synergistic activity in combination with AZT. When tested with AZT at a constant ratio of 1:600, MB 344 was found to be the best candidate for drug therapy due to its very low toxicity, high inhibition of HIV-RT alone and with AZT and good solubility in water.
Introduction

Human Immunodeficiency Virus (HIV) has had a huge global impact on human health since its discovery in the early 1980’s. Since then, scientists worldwide have focused on finding a cure or vaccine for this viral killer. For more than 20 years, HIV has led to over 20 million HIV-related deaths and continues to elude available drugs. With no end to the devastation in sight, the need for an effective treatment grows.

The HIV virion is composed of three parts: the surface glycoprotein, the transmembrane glycoprotein and the central core. The two glycoproteins compose the outer spherical shell and aid in attachment to host cells. The central core contains two copies of the viral RNA genome and the viral enzymes protease, integrase and reverse transcriptase needed for viral replication.

Reverse transcriptase (RT) is a RNA dependant DNA polymerase found only in retroviruses. RT is an essential part of the integration of the RNA genome into the host genome and its subsequent replication. RT has been an important target of current antiretroviral therapies including nucleoside and nonnucleoside RT inhibitors. These inhibitors such as AZT are commonly used in combination with one another due to HIV’s development of resistance to monotherapy. In fact, because of the high replication rate, error prone nature and nonexistent editing function of reverse transcriptase, resistance to current combination therapies has also been found. The need for new reverse transcriptase inhibitors with low toxicity, significant inhibition, and high bioavailability is urgent.
Lavendamycin, a highly toxic antibiotic with anti-viral, anti-bacterial and anti-tumor activity was isolated from the bacterial species, *Streptomyces lavenduale*. Like its related antibiotic, lavendamycin was shown to possess anti-reverse transcriptase activity. Unfortunately, both antibiotics were too toxic to be used *in vivo*. Recently, Dr. Mohammad Behforouz of Ball State University devised a highly efficient method of producing new lavendamycin analogs with low toxicity and a variety of anti-viral capabilities. Several analogs were found to be inhibitory towards HIV-RT.

Previous studies have shown that many of these analogs not only inhibit HIV-RT *in vitro* but also act synergistically with AZT to produce significant inhibition of HIV-RT. In this study, four analogs (MB 72, 76, 97 and 344) are analyzed *in vitro* for their inhibitory capabilities of HIV-RT, both alone and with AZT. First each analog was tested using a checkerboard analysis with four concentrations of AZT (0.007, 0.01, 0.02, 0.04μM) and four concentrations of each analog (0.75, 1.5, 3, 6μM). If inhibitory potential was detected for a specific drug, the optimum ratio with AZT for synergistic inhibition was determined by computer analysis. The optimum ratio was then used for a constant ratio analysis in which increasing concentrations of both drugs at the same ratio were evaluated using the standard RT assay. The resulting data was again analyzed using the Calcsyn® program to give the best ratio and concentrations of the two drugs.
Review of Literature

1. HIV characteristics

The human immunodeficiency virus-1 (HIV-1) has infected more than an estimated 57 million people and killed over 22 million individuals worldwide since the late 1970s. A majority of HIV-1 infected individuals live in developing countries with little or no access to treatment. The highest rates of infection and mortality reside in parts of Asia and sub-Saharan Africa. There is currently no cure for HIV-1 and although treatments are available, they are too expensive for the developing countries where HIV-1 rates are steadily increasing. The need for a vaccine or effective treatment has never been greater (2,3,5).

HIV-1 belongs to the lentivirus family of retroviruses. Lentiviruses also infect a wide range of animal species, especially primates. HIV-1 is basically the same as other retroviruses except for six genes coding for regulatory and accessory proteins that add to the complexity of the HIV life cycle (5). This retroviral genome is encoded by a RNA molecule about 9-kb in length. The HIV-1 virus encodes a total of 15 proteins. Its genome encodes nine open reading frames, three of which encode the structural polyproteins gag, pol, and env. Gag is proteolyzed into four proteins that form the matrix, capsid, nucleocapsid and p6. The two env proteins, surface gp120 and transmembrane gp41, make up the outer envelope and the virion core. The three pol proteins, reverse transcriptase (RT), protease (PR), and integrase (IN), supply the enzymatic functions essential to the viral life cycle. The remaining frames encode six accessory proteins essential for viral replication. Tat and Rev add essential gene
regulatory functions, while $Vpu$, $Vif$, and $Vpr$ are important in viral assembly, disassembly, and down-regulation of CD4. The last protein, $Nef$, also aids $Vpu$ in the down-regulation of CD4 (1,2,5).

2. The HIV genome

As mentioned before, the three major genes of the HIV genome encode for the three structural proteins, which provide the outer membrane proteins and the core protein. The proteins encoded by these three genes, $gag$, $pol$, and $env$, are actually precursor molecules, which are further processed by viral protease to form smaller proteins that will actually provide the viral structure. The processed $gag$ produces the matrix, capsid, nucleocapsid, and p6 core proteins. The matrix is the N-terminal piece of the $gag$ polyprotein and lines the inner surface of the virion membrane of the mature viral particle. The matrix has other functions besides structure. Prior to assembly, the matrix targets the $gag$ and $gag-pol$ precursors to the plasma membrane. The matrix also appears to aid in the transformation of $env$ glycoproteins into viral particles. In addition, the matrix allows infection of non-dividing cell types like macrophages. The capsid forms the core of the virus particle. The N-terminal domain of the capsid protein is important for infectivity, which facilitates viral uncoating. The C-terminal domain assists in assembly through capsid dimerization and $gag$ oligomerization. The nucleocapsid coats the genomic RNA and primarily binds to and delivers full-length viral RNAs to the assembling virion. It is believed that the coating of the genomic RNA is for compaction and protection from various nucleases. p6 is found at the C-terminal of $gag$ and not only aids in viral particle release but also helps to incorporate $Vpr$ during viral assembly (1).
The pol protein (polymerase) is also split into three enzymatic proteins: protease (PR), reverse transcriptase (RT), and integrase (IN). The protease is essential to the formation of mature, infectious virus particles. The protease cleaves various polyprotein sites, facilitating the formation of the matrix, capsid, nucleocapsid, p6, PR, RT, and IN final products. Reverse transcriptase is crucial to the life cycle of the virus because it transcribes a duplex DNA from the viral RNA, allowing for the integration and replication of the viral genome within the host cell, and also serves as a RNA nuclease. Integrase activity follows reverse transcriptase and catalyzes a series of reactions, thereby integrating the viral genome into the host cell’s chromosome (1).

The env gene codes for a fusion-incompetent precursor protein, gp160, which is then proteolytically cleaved into two subunit viral surface proteins, gp41 and gp120. Both subunits are very important for the binding and fusion of the virus to the host cell. The surface glycoprotein (gp120) binds to cellular receptors, while the transmembrane glycoprotein (gp41) is involved in the fusion of the viral and cellular membranes after receptor binding (1,7).

The remaining six genes code for six accessory proteins that play a smaller, but still very important role in the life cycle of HIV. Vpr, vif, vpu, tat, rev, and nef help to increase the complexity of the virus. Viral protein r (vpr) is believed to play an important role in viral disassembly and transport of the pre-integration complex (PIC) to the host cell nucleus (2). Following entry and the uncoating of the virus, vpr transports the nucleoprotein complexes (PIC) to the host cell nucleus. These nucleoprotein complexes most likely consist of RT, IN and the matrix; viral components needed for the next step, integration (1). Virion infectivity factor (vif) is either directly or indirectly involved in
virus assembly and is essential for viral replication in vivo and some cultured cell types. *Vif* is needed by HIV to establish infection in certain cell cultures such as H9 T cells, macrophages, and peripheral blood lymphoid cells. These cells are referred to as non-permissive, while cell types that allow viral infection without *vif* are designated permissive (6). Viral protein u (*vpu*) down-regulates the expression of the cellular receptor, CD4, on the cell surface (2). In order to prevent newly synthesized gp120 and gp41 from remaining in the endoplasmic reticulum through interactions with CD4 molecules, *vpu* supports the degradation of CD4 in these complexes. The CD4 degradation allows the *env* proteins to proceed to the cell surface and begin the assembly of viral particles. *Vpu* may also be involved in virion release (1). The transactivator of transcription (*tat*) is vital to viral replication because it influences the rate of transcription of the DNA form of the virus (provirus). Transcriptional initiation and elongation are mediated by RNA polymerase II, which is regulated by the HIV promoter located in the 5' long terminal repeat (LTR). *Tat* is capable of enhancing transcriptional elongation and the processivity of transcribing polymerases through the interaction with this promoter. *Tat* also increases the production of viral mRNAs 100-fold (1,5). The regulator of expression of viral proteins (*rev*) binds to a cis-acting RNA target, the Rev response element (RRE), found in all unspliced viral transcripts and targets them for nuclear export. Normally, unspliced cellular RNAs are either completely spliced or degraded in the nucleus (5). The last protein, negative factor (*nef*), reduces the level of cellular CD4 with *vpu*. *Nef* assists in the transference of CD4 from the cell surface and golgi apparatus to lysosomes. This *nef*-facilitated degradation of CD4 once again prevents improper interactions of the CD4 with *env* proteins in the ER. *Nef* has also been shown to down-
regulate the expression of MHC I molecules which may evade infected cells from
detection and death by cytotoxic T cells (1).

The nine gene viral genome is flanked by a 5' and 3' long terminal repeat (LTR)
sequence. The 5' LTR contains the HIV promoter, a number of regulatory elements, and
several sites for cellular transcription factors such as NF-κB, Sp1, and TBP.

**Figure 1: Organization of the HIV-1 genome and virion** (1)

3. HIV pathogenesis

The major problem with HIV infection is that despite a strong humoral and cell-
mediated immune response, the immune system is only able to partially control the
infection. There are actually two pathogenic forms of HIV-1, distinguished by their cell
tropism. HIV-1 strains that are primarily transmitted by sexual contact are called M-
tropic viruses. Also known as NSI (non-syncytium inducing) primary viruses, this form
can infect and replicate in macrophages as well as primary CD4+ T cells. T-tropic
viruses, also known as SI (syncytium inducing) primary viruses, are the HIV-1 viruses
that only infect and replicate in helper T cells; both primary CD4+ T cells and established CD4+ T cells in vitro. M-tropic HIV-1 strains are found in about 90% of sexually transmitted HIV. Although T-tropic transmission is rare, once this strain becomes established they are more virulent and cause faster disease progression (22).

The tropism of HIV-1 is dictated by the type of chemokine receptor used. CCR5 is expressed on macrophages and therefore used by M-tropic viruses as their coreceptor. CXCR4 is expressed on T cells and is used as a coreceptor for T-tropic viruses. Primary T cells express both CCR5 and CXCR4 on their surface and are consequently infected by M-tropic, T-tropic, and dual-tropic viruses (7). Although HIV does need these coreceptors, CD4+ is still the major receptor for the virus.

CD4+ T cells and CD8+ T cells are the principle cell types involved in the pathogenesis of HIV. Since the CD4+ T cells are the primary targets for HIV, the depletion of these lymphocytes is considered to be the principle event responsible for the characteristic immune deficiency in HIV patients. CD8+ cytotoxic T lymphocytes are believed to play an important role in the control of HIV infection through their cytotoxic activity and the release of soluble factors to suppress viral replication. Unfortunately, CD8+ T cells are also thought to be susceptible to viral infection, which helps to explain why HIV has such a damaging effect on the immune system. Although not yet proven, in vitro experiments have shown that CD8+ T cells can become double-positive T cells (CD4+/CD8+) through stimulation by the T cell receptor complex. In other words, the infected CD4+ T cells cause the CD8+ cells to express the CD4+ antigen on their surface, consequently allowing CD8+ T cells to be infected and affected by HIV (9,10). The different types of coreceptors used and the different cell types that can be infected, allows
HIV numerous ways to infect cells, avoid viral inhibitors, and deplete the immune system.

4. Life cycle of HIV

1) Virus entry

Viral entry into the host cell requires that the virus bind to a cell surface receptor, fuse with the cell, and then deliver a virion into the cytoplasm of the uninfected cell. The pathway of virus entry into a cell requires viral glycoproteins, coreceptors, and the cell surface molecule CD4.

The proteolytic products of gp160, produced by the \textit{env} gene, mainly facilitate viral membrane fusion. The surface subunit, gp120, remains noncovalently associated with the transmembrane subunit, gp41. gp120 is responsible for binding the cell surface receptor, CD4. CD4 is a molecule commonly found on human T cells but can also be found on other cell types such as monocytes and macrophages (4,9). The joining of gp120 and CD4 is believed to activate the membrane fusion ability of gp41. However, the binding of CD4 is insufficient to activate membrane fusion. The fusion requires gp120 to engage a second receptor following CD4 binding. This coreceptor can include any of the seven transmembrane-domain, G-protein-coupled receptors for chemokines. Chemokine receptors CCR5 and CXCR4 are believed to be the most common coreceptors for HIV-1 entry (4). Chemokine receptors normally function as cell surface proteins that bind small peptides called chemokines, which play an important role in host immune surveillance and inflammatory response. While the outer membrane portion binds the chemokine, the inner membrane portion is involved in cell signaling (3, 11).
After the conditions of specific pH and CD4 and coreceptor binding are met, membrane fusion may proceed.

**Figure 2: The replication cycle of HIV-1(1)**

Membrane fusion is a complicated mechanism. Following the fusion-activating conditions, certain conformational changes occur allowing the fusion peptide component of gp41 to become unexposed. The fusion peptide release may be by the spring-loaded mechanism or just becomes uncovered through the movement of gp120. These movements result in the formation of the transient prehairpin intermediate. This intermediate spans two membranes: the viral transmembrane region and the host cell membrane where the fusion peptide is now located. The prehairpin intermediate then becomes a trimer of hairpins, facilitating membrane fusion (7).

The completion of membrane fusion allows for the virus to infection the host cell. Successful infection requires that the nucleoprotein core, consisting of the viral genome
and proteins, be delivered into the cytoplasm. This nucleoprotein core contains all the components necessary for the continuation of the HIV life cycle through further production of new virions.

2) Reverse transcriptase

In order for the virus to be replicated by the host cell it must be integrated into the host chromosome. Before viral integration can occur, the viral RNA genome must be transcribed into duplex DNA. The viral enzyme, reverse transcriptase, catalyzes this conversion from genomic RNA to a double-stranded proviral DNA copy. This change occurs through the actions of three components: DNA-dependent DNA polymerase, RNA-dependent DNA polymerase, and ribonuclease H (RNase H). All three elements are provided by the enzymatic activities of reverse transcriptase. Reverse transcription of the viral RNA begins at the 3' end of a tRNA-Lys primer annealed to the primer binding site in the 5' region of the genomic RNA. Although RT can use other tRNAs, reverse transcription is most effective with tRNA-Lys. After the tRNA initiation, reverse transcription involves two DNA strand transfer reactions in which RNA-dependent DNA polymerase creates a DNA strand from the viral RNA template, followed by the use of DNA-dependent DNA polymerase to produce the complementary DNA strand. The RNase H component of RT facilitates the cleavage of the RNA portion of the RNA-DNA hybrid so that the plus and minus DNA strand may join to form the DNA duplex (1,12).

Reverse transcriptase is an asymmetric heterodimer consisting of p51 and p66 polypeptides originating from the pol protein. Both p51 and p66 subunits contain a polymerase domain composed of four subdomains: fingers, palm, thumb, and connection. In addition, the p66 subunit contains the RNase H domain. Although the
subdomains of both subunits are identical in sequence of amino acids, the polymerase subdomains of the subunits are oriented differently forming a large active site cleft in p66 and an inactive, closed structure for p51. Reverse transcriptase has also been found to undergo large conformational rearrangements, especially changing the position of the p66 thumb, upon nucleic acid binding. These conformational changes of the RT molecule may be important in moving RT along the nucleic acid chain or to correctly position RNase H when needed (1,12).

**Figure 3: Structure of HIV-RT (12)**

The reverse transcriptase molecule is believed to be an ideal target for drug therapy. Two types of RT inhibitors have already been developed. Nucleoside analog inhibitors, such as AZT, bind to the polymerase active site, interrupting chain elongation. Non-nucleoside inhibitors, such as nevirapine, bind to a hydrophobic pocket near the polymerase active site, causing allosteric changes that inactivate RT. Unfortunately, the
binding of these two drug types is very specific so resistance can be conferred by as little as one amino acid change (1,12,15).

3) Viral integration

After reverse transcription has produced the duplex DNA, viral integrase recognizes the LTR sequences and catalyzes three reactions that insert the DNA precursor into the host genome. The viral DNA precursor contains a complete plus strand and a discontinuous minus strand that is probably completed by host enzymes after integration. In the first step, integrase removes two nucleotides from the 3' end of each viral DNA strand, leaving projecting cytosine-adenine-OH ends. The second step involves the 3' ends being joined covalently to the 5' ends of the host DNA. In the third step, unpaired nucleotides at the viral 5' ends are removed and the ends are joined to the 3' host DNA ends. The final result is an inserted provirus with five base-pair duplications of the target site DNA on each side (1,8).

Integration can have many target sites within the host genome. In vitro studies have shown that viral integration may have a preference for sites of kinked or distorted DNA, common to nucleosomes. It has also been found that at least two host cell proteins, HMG-I(y) and BAF, increase the efficiency of integration (1,8).

4) Viral protein expression

The expression and production of the viral proteins from the newly integrated provirus requires the use of the host cell’s protein production mechanisms and the virus’ regulatory proteins tat and rev. Host transcription of the viral DNA produces spliced
viral mRNAs, which produce all nine viral polyproteins following host translation of the viral mRNA (1).

Viral protein expression begins by viral transcripts being expressed from the promoter in the 5' LTR. *Tat* regulates and greatly increases the rate of transcription. While cellular RNA polymerase II and transcription factors such as NF-κB, Sp1, and TBP oversee the initiation of transcription, *tat* is required for elongation to proceed. In its absence, polymerase does not transcribe more than a few hundred nucleotides. *Tat* has a common transcriptional activator structure, including an activation domain and a RNA binding domain. In order for *tat* to function correctly, a *tat*-activation region (TAR) must be present in the form of a bulged, RNA stem-loop structure found at the 5' end of the viral mRNA. Following cellular (RNA polymerase II and transcription factors) initiation of transcription, *tat* complexes with cellular CycT and Cdk9 and then binds to the TAR on the small mRNA transcript. The Cdk9 then hyperphosphorylates a component of the RNA complex, allowing elongation to proceed (1,5).

*Rev* facilitates the exportation of the transcribed viral mRNA from the nucleus to the cytoplasm, where it can then be translated. *Rev* binds to a cis-acting RNA target, *rev* response element (RRE), present in all unspliced viral transcripts through its NH2 domain. After binding, *rev*-rev multimerization occurs, forming a complex that acts as a nuclear exportation signal. Once in the cytoplasm, the associated cellular exportation proteins disassociate and *rev* returns to the nucleus, leaving the viral mRNA to be translated (5). Viral mRNAs are then translated in the cytoplasm, producing *gag* and *gag-pol* polyproteins. *Env* mRNA is translated at the endoplasmic reticulum. The translation of *nef* is an important event because it down-regulates the surface expression of CD4,
allowing the \textit{gag} and \textit{gag-pol} polyproteins to localize on the cell membrane and preventing CD4 from interfering with the newly synthesized \textit{env} proteins (1).

5) Virus assembly and budding

The matrix is responsible for the localization of the \textit{gag} and \textit{gag-pol} polyproteins to the plasma membrane before virus assembly. The matrix consists of two main parts: basic residues and an N-terminal myristate group. The matrix forms a trimeric structure, believed to be responsible for virus assembly. When matrix residues responsible for trimerization have been mutated the ability to assemble has been lost. Other residues are also important for membrane localization. The nucleocapsid, like the matrix, also comes from the gag polyprotein. The main function of the nucleocapsid during assembly is to bind to the packaging signal and deliver viral RNAs to the assembling virion (1).

Budding of the viral particle begins once the core particle has been assembled from the gag and gag-pol polyproteins, vif, vpr, nef, and genomic RNA. After the assembly of the core particle, the gag and gag-pol polyproteins are further processed into the matrix, nucleocapsid, capsid, p6, protease, RT, and integrase. Before the budding particle can leave the cell surface as a mature virion, it must first be coated by the surface and transmembrane proteins. These proteins are products of the env polyprotein, which is initially complexed with CD4 in the endoplasmic reticulum. Vpu then promotes CD4 degradation, releasing env and transporting it to the cell surface. The cell surface CD4 must then be degraded by nef at the site of viral budding so that the surface and transmembrane proteins may coat the budding virus. Once coated, the virus particle is
released from the host cell and undergoes the process of maturation. Viral maturation is accomplished through protease cleaving the gag and gag-pol polyproteins and vif ensuring intracellular and endogenous reverse transcription and correct viral stability and morphology. Once the virion is mature it is ready to infect another host cell, completing the life cycle of HIV (1,6).

5. Reverse Transcriptase Inhibitors

The reverse transcription enzyme is unique to retroviruses. It is also very important to these type of viruses because it facilitates the production of a DNA copy of the viral RNA genome that can be inserted into the host cell DNA for viral replication. The uniqueness and importance of reverse transcriptase make it an excellent target for anti-viral drugs. Currently, RT inhibitors can be divided into two classes, nucleoside and non-nucleoside inhibitors.

1) Nucleoside inhibitors

Nucleoside inhibitors (NRTIs) were the first to be discovered, before the structure of RT had even been determined. These inhibitors terminate chain elongation through the nucleoside analog substrates blocking in the substrate-binding site. Currently approved nucleoside inhibitors include zidovudine (AZT), didanosine (ddI), lamivudine (3TC), stuvudine (d4T), zalcitabine (ddC), and abacavir (ABC) (14,13,15,18,21).

In the case of AZT, its 3'-azido group prevents the formation of a 3'-5' phosphodiester bond, terminating the viral DNA chain. AZT was produced in 1987 as the first generally available AIDS drug. The popular drug is administered orally and has a high bioavailability and short intracellular half-life. Didanosine is an analog of inosine
and like AZT, its active form is a triphosphate created by cellular enzymes. It also has a longer half-life than AZT, allowing for only once a day dosing. Zalcitabine is related to didanosine and is active against HIV at very low concentrations. Unfortunately, it has a very short half-life, so multiple doses are required per day. Stavudine is a modification of thymidine that is well tolerated with few side effects in patients (14).

4) Non-nucleoside inhibitors

While nucleoside inhibitors were developed to interfere with the mechanism of RT, non-nucleoside inhibitors generally interfere with the structure of the enzyme. Non-nucleoside inhibitors (NNRTIs) are chemically diverse, hydrophobic compounds that comprise over 30 different classes. Unlike NRTIs, non-nucleoside inhibitors do not require intracellular metabolism for activity and frequently inhibit only HIV-1. They are characterized by their noncompetitive inhibition of RT activity and are primarily low or noncytotoxic. NNRTIs inhibit reverse transcriptase activity by binding to a hydrophobic pouch close to the p66 active site. The binding subsequently causes allosteric changes that prevent reverse transcription of the viral RNA genome. There are currently three non-nucleoside inhibitors approved for use against HIV: nevirapine, delavirdine, and efavirenz (12,13,14,15,18,21).

Nevirapine was the first NNRTI to gain approval. The drug has a high bioavailability in its oral form and is able to induce its own metabolism. Delaviridine has a short half-life and requires many doses a day. Efavirenz is very effective when used in combination therapy, but resistance to it is easily gained when used alone (14).
6. Protease inhibitors

Another group of approved drugs inhibit the viral enzyme protease. Protease is required for proper virion assembly and maturation. Inhibition of protease leads to the production of immature, noninfectious virus particles. There are currently six protease inhibitors approved for use: saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, and lopinavir. The availability of protease inhibitors has changed the course of the disease and considerably reduced HIV's morbidity and mortality (14,21).

7. Combination therapy

Although many inhibiting drugs have been developed, mutant HIV strains resistant to all of them have been identified. The development of resistance is due to both the high replication rate of the virus and the error prone nature of HIV RT, leading to mutation. Individuals infected with HIV produce over 1 billion virions a day. The HIV RT has no editing function and therefore makes an error for every 2,000-5,000 nucleotides that are polymerized. It is not surprising then that any of the current drugs used as monotherapy only offer a short-term benefit. Resistance developed through mutation is often observed only a week or two after the start of therapy. Current HIV and AIDS treatment now relies on combination therapy, using nucleoside inhibitors with non-nucleoside inhibitors or protease inhibitors. Combinations of retroviral drugs have shown a dramatic reduction of AIDS-related morbidity and mortality. Combination therapy of RT and protease inhibitors has effectively suppressed viral replication to minute levels for long periods of time and decreases the ability of viruses to mutate to resistance to several drugs at once. NNRTIs are especially easy to gain resistance against. Since their
binding site is very specific, a single amino acid change in that site can render the drug useless. Therefore, NNRTIs must be used in combination therapy with other drugs that together prevent the emergence of HIV strains resistant to NNRTIs (13,14,16,17,18). Proper combination strategies are selected by their ability to show synergy, while at the same time, maintaining low toxicity (21).

In combination therapy, synergy is defined as yielding an amount of inhibition activity above the level of additive interaction. In other words, the amount of inhibition of two drugs together is greater than the sum of the amount of inhibition of each drug alone. NNRTIs commonly exhibit synergistic inhibition of HIV when used in combination with NRTIs (16,20,21).

7. Lavendamycin

Lavendamycin is an antibiotic produced by *Streptomyces* that has shown considerable biological activity and anti-tumor capabilities. Furthermore, lavendamycin and another *Streptomyces* antibiotic, streptonigrin, have been shown to contain strong anti-reverse transcriptase activity against HIV-RT. Regrettably, these antibiotics are highly toxic to the human body, thought to be due to disruption of DNA synthesis. Recently, M. Behforouz of Ball State University has synthesized several novel lavendamycin analogs using short, efficient synthetic methods that produce high product yields (24,25). These analogs have so far been shown to have significant anti-reverse transcriptase activity, possible synergistic activity, and low cellular and animal cytotoxicity (26).
8. Purpose of this study

As of 2000, it is estimated that 30-50% of HIV/AIDS patients are failing combination therapy due to the development of resistance and noncompliance with dosing schedules. The development of new HIV inhibitors, with low side effects and high bioavailability, are greatly needed to provide more combination therapy options and to keep the upper hand on the battle with HIV (18). The purpose of this study is to further evaluate novel lavendamycin analogs in combination with AZT for their synergistic inhibition of HIV-RT in vitro. The inhibition activity of these analogs will be determined though the use of four concentrations of AZT (0.04, 0.02, 0.01, and 0.007μM) in combination with four concentrations of lavendamycin analogs (MB) (6, 3, 1.5, and 0.75μM). The various combinations of the two drugs will be tested through a checkerboard analysis and the resulting data will be analyzed for signs of synergistic, additive, or antagonistic activity using the CalcuSyn® computer program (29). Promising drug combinations will then be tested using the optimum synergistic ratio of the two drugs at decreasing and increasing concentrations. This study will hopefully reveal several MB analogs as potential non-nucleoside reverse transcription inhibitors to be further tested for their possible use in anti-HIV combination therapy.
Materials and Methods

1. Determination of Anti-HIV Reverse Transcriptase Activity

1) Assay reagents

   a) 8X reaction buffer- 800mM Tris HCL, 40mM MgCl2, 480mM NaCl, and 40mM of dithiothreitol, at a pH of 8.0.
   
   b) Poly (rA)-oligo (dT)- purchased from Amersham Pharmacia Biotech, Piscatway, NJ (25units/1366µg) and diluted into 34.15 ml of buffer (0.01M Tris HCl, 0.1M NaCl at pH 7.5) to give a 40µg/ml stock solution, which was stored at -80°C. A final concentration of 4µg/ml would result in the reaction mixture.
   
   c) [Methyl-3H]-deoxythymidine 5'-triphosphate ([3H] TTP)- purchased from ICN Biomedicals, Costa Mesa, CA (1mCi/ml).
   
   d) Wash solutions- 1) 5% trichloroacetic acid in 0.01M sodium phosphate monobasic, 2) 0.6M sodium chloride in 0.06M sodium citrate, 3) double distilled, deionized water, and 4) 95% ethanol.
   
   e) Scintillation fluid, type E- purchased from Sigma Chemical Company, St. Louis, MO.

2) Drugs

   a) AZT Triphosphate- purchased from Moravek Biochemical, Brea, CA (5µM in 1ml).
b) Lavendamycin analogs- obtained from Dr. Mohammed Behforouz, Ball State University Chemistry Department (Table 1). Each analog, except MB 344, was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 1μg/ml. MB 344 was dissolved in RPMI-1640 culture media with 5mM Hepes buffer to a concentration of 1μg/ml. Each analog stock solution was diluted with double distilled water to 15, 6, 3, 1.5, and 0.75μM for use in the final reaction mixture.

3) HIV Reverse Transcriptase Enzyme
   a) Recombinant HIV-RT from *E.coli* plasmid, pRC-RT (500units/vial)- purchased from Worthington Biochemical Corp., Frenhold, NJ, aliquoted to 150units/ml, and stored at -80°C.
   b) HIV-RT storage solution- 100mM potassium phosphate buffer (33mM KH2PO4, 67mM K2HPO4 3H2O) at pH 7.1, 1mM dithiothreitol (DTT), and 10% glycerol at pH 7.1.

4) Anti-HIV-RT Activity Assay
   The inhibition of HIV-RT activity was measured by a modification of Take and Okada et.al and Robins et.al (27,28).

   The assay was performed as follows: The lavendamycin analogs were dissolved in DMSO at a concentration of 1μg/ml, sometimes with sonification and mild heat, and then were serially diluted with double distilled water to achieve concentrations ranging from 15 to 0.75μM. For each assay, the final reaction mixture containing 8X buffer,
ddH2O, 3[H]TTP, template primer, and HIV-RT was premixed in a 15ml sterile tube in the required amounts for each experiment. The tube was then mixed by vortexing and placed on ice. The inhibitors, MB analogs or AZT, were added to the appropriate wells in a 24 well microtiter plate and ddH2O was added to each well to bring the total volume in all wells to 103µl. 195µl of the final reaction mixture was then added to each of the wells and the plate was gently mixed. The plate also included triplicate sets of three wells, each with no inhibitors, to serve as enzyme controls. The plate was then incubated for one hour at 37°C. Following incubation the reaction was stopped by the addition of 0.1M EDTA. Each well was then mixed by pipeting and 15µl from each well was placed on a 1cm square of Whatman DE81 ion exchange paper. Three squares were each soaked with the 15µl solution for each well. After drying, each filter paper was washed for 8 minutes three times with 5% TCA-NaHPO4 12 H2O, three times with 0.6M NaCl-0.06mM Na Citrate, 1 time with double distilled water, and 1 time with 95% ethanol. Each 8-minute wash consisted of a vial containing the filter papers for each well and 4-5 ml wash solution, being shaken by a shaker. After each wash, the wash solution was removed by vacuum. After drying, each filter paper was placed in a fresh, separate vial with 5ml of scintillation fluid – type E. The amount of 3[H]-TTP uptake (noting successful HIV-RT synthesis) was measured as counts per minute on a Beckman liquid scintillation counter. Each experiment for a particular MB analog was repeated at least twice to verify results.
Figure 4: Assay Procedure

- For each triplicate 298μl Reaction Mixture: 8X buffer........39.2μl
  
  DdH2O..........121.8μl

  $^3$H-TTP........2μl

  Template primer..30μl

  HIV-RT..........2μl

  AZT and/or

  MB analogs........Various depending on concentration

- Incubate at 37°C for one hour

- Add EDTA to stop reaction

- Add 15μl from each well to 1cm square filter paper (in triplicates for each well)

- Wash filter papers for 8 minutes for each washing solution

- Let filter paper air dry

- Add filter paper to 5ml scintillation fluid

- Count $^3$H-TTP uptake using scintillation counter
## TABLE 2
Lavendamycin Analogs

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>R¹</th>
<th>R²</th>
<th>R³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lavendamycin</td>
<td>NH₂</td>
<td>CO₂H</td>
<td>CH₃</td>
</tr>
<tr>
<td>MB-21</td>
<td>CH₃CO</td>
<td>CO₂CH₃</td>
<td>CH₃</td>
</tr>
<tr>
<td>MB-61</td>
<td>CH₃CO</td>
<td>CO₂H</td>
<td>H</td>
</tr>
<tr>
<td>MB-72</td>
<td>CH₃CH₂CH₂CO</td>
<td>CO₂CH₂CH₂CH₂CH₃</td>
<td>H</td>
</tr>
<tr>
<td>MB-76</td>
<td>CH₃CO</td>
<td>CONH₂</td>
<td>H</td>
</tr>
<tr>
<td>MB-83</td>
<td>H</td>
<td>CO₂C₈H₁₇-n</td>
<td>H</td>
</tr>
<tr>
<td>MB-97</td>
<td>H</td>
<td>CONH₂</td>
<td>H</td>
</tr>
<tr>
<td>MB-344</td>
<td>CH₃CO</td>
<td>CONHCH₂CH₂OPO(OH)₂</td>
<td>H</td>
</tr>
</tbody>
</table>
Results

1. Combined effect of lavendamycin analogs and AZT on HIV-RT

Today’s treatment of HIV is dependant on combined drug therapies using ideal anti-reverse transcriptase drugs. An ideal drug must significantly inhibit reverse transcriptase at a concentration low enough to be non-toxic. There are three drug interactions possible when multiple drug therapy is concerned: an antagonistic, additive, or synergistic effect. In an antagonistic interaction, the drugs interfere with each other to produce a total effect that is significantly lower than the additive effects of each drug alone. In an additive interaction, the drugs act independently of each other to produce a total effect that is about equal to the additive effect of each drug alone. Synergistic interaction is ideal for the use of multiple drugs because the drugs combine to give a total effect that is higher than the additive effects of each drug action alone. Synergism allows for high inhibition of reverse transcriptase while also maintaining low drug concentrations.

First, the combined effect of the lavendamycin analogs with AZT-triphosphate was analyzed using a checkerboard method with a range of concentrations of both drugs. The results of this assessment are presented in Table 3 and show two separate trials for MB 97 and MB 344, and one trial for MB 72 and MB 76. MB 72 and MB 76 were done only once as a follow up on previous work. The results were then analyzed using the method of Chou and Talalay and characterized by the combination indices (CI) for each two drug combination (29). The combination index is derived from a computerized calculation of the slope of the median-effect plot and the x-intercept of the plot. These
graphs of the data give the dose-effect curve and the potency of each drug, both alone and in combination, respectively. A sample median-effect plot and dose-effect curve for the MB 344 and AZT-triphosphate combination is shown in Figure 4.

The results of the checkerboard analysis show that combinations of all the analogs with AZT-triphosphate exhibit synergistic effects, along with additive and antagonistic effects. The greatest amount of synergism among different concentrations was detected in the analogs MB 344 and MB72.

MB 344 and MB 72 were further analyzed to find the dose reduction index of the drug combinations. Once again the method of Chou and Talalay was used to find the dose-effect curves for each drug as well as for their combination with AZT-triphosphate at constant-ratios. The constant ratio analysis allowed for the determination of the combination indices (CI) at a 50% and 75% effect level and the dose reduction index (DRI) at 50% inhibition for both MB 344 and MB 72, shown in Table 4. The DRI is a measure of how much each drug can be reduced in a synergistic combination and still give a desired inhibition. As seen in the checkerboard analysis, MB 344 displayed the best results in the constant ratio analysis (1:600 ratio) with very strong synergism (CI<0.1) for both the 50% and 75% effect levels and a DRI of more than 100 times for both the analog and AZT-triphosphate at 50% inhibition.
Table 3. Combination Indices Determined for Lavendamycin Analogs Performed by Checkerboard Analysis

Lavendamycin Analogs (μM)*

<table>
<thead>
<tr>
<th></th>
<th>72</th>
<th></th>
<th>76</th>
<th></th>
<th>97</th>
<th></th>
<th>344</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.75</td>
<td>1.5</td>
<td>3</td>
<td>6</td>
<td>0.75</td>
<td>1.5</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Trial 1</td>
<td>0.54</td>
<td>0.62</td>
<td>0.65</td>
<td>0.44</td>
<td>1.16</td>
<td>1.08</td>
<td>1.07</td>
<td>1.14</td>
</tr>
<tr>
<td>Trial 2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>AZT (μM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial 1</td>
<td>1.03</td>
<td>0.90</td>
<td>0.64</td>
<td>0.56</td>
<td>1.28</td>
<td>0.96</td>
<td>1.07</td>
<td>0.98</td>
</tr>
<tr>
<td>Trial 2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Four different lavendamycin analogs were combined in different ratios with AZT-triphosphate and added to HIV-RT enzyme assays in triplicate. Two separate trials were performed for each drug unless otherwise noted “not done” (ND). The combination index (CI) of each drug combination was analyzed by the method of Chou and Talay (29). CI values <1 indicate synergism, 1 indicates additive effects and >1 indicates antagonism. CI values <0.7 are considered to be synergistic and are shown in bold. Values ≥0.71 and ≤0.9 are considered to be moderately or slightly synergistic and are shown in italics.
Table 4. Two Drug Combination of AZT and lavendamycin analogs at constant molar ratios

<table>
<thead>
<tr>
<th>Molar Ratio</th>
<th>CI at HIV-RT Inhibition of:</th>
<th>Dose Reduction(^2) Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50%</td>
<td>75%</td>
</tr>
<tr>
<td>AZT:MB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>1:600</td>
<td>0.45 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>1:857</td>
<td>0.39 ± 0.06</td>
</tr>
<tr>
<td>344</td>
<td>1:600</td>
<td>&lt;0.1 ± 2.24</td>
</tr>
<tr>
<td></td>
<td>1:857</td>
<td>2.17 ± 3.68</td>
</tr>
</tbody>
</table>

\(^1\) Combination index at the effect level of 50% and 75% as calculated by the method of Chou and Talay. Additive effect (CI=1), synergism (CI<1), or antagonism (CI>1) (29).

\(^2\) Dose Reduction Index is a measure of how much the dose of each drug in a synergistic combination may be reduced at the 50% effect level compared with the doses of each drug alone (30).
Figure 4. Example of median-effect and dose-effect plots for MB 344
Discussion

Combination therapy is now essential for the treatment of patients afflicted with HIV. As HIV is now becoming resistant to current combinations of drugs, the need for novel medicines has never been so great. Unfortunately, rapid viral mutation, drug toxicity, large drug doses and patient non-compliance make it hard to develop new treatments. The ideal drug would be highly potent and synergistic with other drugs, easily produced, have a low toxicity and be required in small doses.

Lavendamycin analogs were initially tested because of their relatedness to streptonigrin, a reverse transcriptase inhibiting but highly toxic antibiotic. Lavendamycin is also toxic but the novel analogs produced by Dr. Mohammad Behforouz have exhibited low toxicity in both murine and human cells. Previous studies have shown that for lavendamycin analogs, the mean inhibitory concentration required to inhibit 50% of HIV-RT (IC50) is well below the mean concentration required to kill 50% of human and murine cells (CC50). The IC50 for MB 72 and MB 76 was much less than the CC50 of the drugs on human and murine cells. MB 344 had an IC50 even lower than its CC50. However, MB 97 was found to have an IC50 much greater than its CC50 for both cell types, so it was too toxic to actually be considered for drug use (21). Therefore, MB 344, 72 and 76 could be used in human HIV drug therapy if found to be potent enough.

The four lavendamycin analogs were chosen based on their performance in previous studies of both synergistic inhibition of HIV-RT and cellular cytotoxicity. Although all four analogs that were tested showed synergistic capabilities, only the most promising analog should be further tested due to HIV’s devastating capabilities. MB 76
showed barely any synergistic potential, while MB 97 did not work well in experimental tests and is highly toxic. Although MB 72 did exhibit good synergistic activity, MB 344 is clearly the best candidate with its high synergism at 50% and 75% effect levels and drastic DRI at the 1:600 molar ratio with AZT-triphosphate. MB 344 also showed to have an IC50 value almost 10 times less than human and murine CC50 values (21). MB 344 was also found to solubilize in water much better than most other analogs, which could possibly explain its effectiveness and make drug production easier.

In summary, testing has shown that at least one of the new lavendamycin analogs, MB 344, has characteristics of a promising new drug. The water-soluble drug has exhibited great inhibitory activity alone and in combination and very low toxicity in animals. This potential candidate may prove to be a potent addition to the nonnucleoside reverse transcriptase inhibiting HIV drugs, following further testing.
References


