The Effects of Newly Synthesized Compounds on the HIV-1 Reverse Transcriptase and Blood Cells

By

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ABSTRACT

Acquired Immunodeficiency Syndrome (AIDS) is a result of replication of Human Immunodeficiency Virus (HIV-1) in an infected host. Reverse transcriptase (RT) enzyme of HIV-1 is a multifunctional enzyme in the life cycle of the virus. Even though many compounds have been developed against different aspects of HIV-1, RT enzyme is a prime target for the development of drugs against HIV-1 because eucaryotic cells do not have RT activity. In order to develop new therapeutic agents against HIV-1, nineteen newly synthesized compounds were analyzed for their effects on inhibition of RT activity as well as their effects on viability, proliferation, and activity of peripheral blood mononuclear cells (PBMC). Finally, mutagenic effects of the compounds were investigated.

Results indicated that AVM 002 and AVM 014 were the most promising compounds with 51% and 43% inhibitory effects, respectively on RT enzyme at 100μM concentration. The compounds AVM 001, AVM 010, AVM 011, AVM 015 and AVM 019 also showed inhibitory effects between 24% and 40% at 100μM concentration. AVM 002 did not cause any toxic effect on cell viability, proliferation and activation until 500μM concentration. Similar to AVM 002, AVM 014 did not show any toxic effect on the cell viability until 500μM and 1000μM at the and of 24 hour incubation; however, AVM 014 at 500μM and 1000μM resulted in 2-fold decrease in the cell viability after 48 and 72 hour incubation, compared to the control. Unlike AVM 002, AVM 014 gave rise to 3,4-fold decrease in cell activity at the end of second and third day incubation. Moreover, among all of the tested compounds, AVM 010 was the most toxic at 500μM and 1000μM compared to the control. Furthermore, all the compounds did not show any mutagenic effect on *Salmonella typhimurium* TA 100 and TA 102 strains.

In summary, the results indicate that AVM 002 and AVM 014 is the best candidate to be improved in order to reach higher RT enzyme inhibitory effect and decreased cytotoxicity profile by slight modifications in compound structure.

ÖZ

Acquired Immunodeficiency Syndrome (AIDS), Human Immunodeficiency Virus (HIV-1)' in enfekte konakçıda çoğalmasının sonucudur. HIV-1'in Revers transkriptaz (RT) enzimi virüsün hayat döngüsünde çok fonksiyonlu bir enzimdir. HIV-1'in farklı durumları için pek çok bileşik geliştirilmiş olmasına rağmen, ökaryotik hücrelerin RT aktivitesine sahip olmamaları, RT enzimini HIV-1'e karşı ilaç geliştirilmesinde en önemli hedef yapar. HIV-1'e karşı yeni terapatik bileşiklerin geliştirilmesi amacıyla yeni sentezlenmiş ondokuz bileşiğin RT inhibisyon etkileri ve aynı zamanda periferal kan mononüklear hücrelerinin (PBMC) canlılığına, bölünmesine ve aktivasyonuna olan etkileri analiz edildi. Son olarak bileşiklerin mutajenik etkileri araştırıldı.

Sonuçlar AVM 002 ve AVM 014'ün 100µM konsantrasyonda sırası ile %51 ve %43 RT inhibisyonu ile en ümit verici bileşikler olduğunu göstermiştir. AVM 001, AVM 010, AVM 011, AVM 015 ve AVM 019, 100µM konsantrasyonda %24-%40 arasında inhibisyon etkisi göstermişlerdir. AVM 002, 100µM ve 500µM konsantrasyonlara kadar hücre canlılığı, aktivasyonu ve bölünmesi üzerine herhangi bir toksik etkiye neden olmamıştır. AVM 002'ye benzer şekilde, AVM 014 500µM ve 1000µM konsantrasyonlara kadar, 24 saatin sonunda hücre canlılığı üzerinde toksik etki göstermemiş, fakat AVM 014'ün 500µM ve 1000µM konsantrasyonları 48 ve 72 saatlerin sonlarında kontrol ile karşılaştırıldığında 2 kat düşüşe neden olmuştur. AVM 014, AVM 002'den farklı olarak ikinci ve üçüncü günlerin sonunda 500µM ve 1000µM konsantrasyonları hücre aktivasyonunda 3,4 kat azalmaya neden olmuştur. Dahası, tüm test edilen bileşikler içinde AVM 010, 1000µM konsantrasyonu kontrol ile karşılaştırıldığında en toksik bulunmuştur. Ayrıca test edilen bileşikler *Salmonella typhimurium* TA 100 ve TA 102 türleri üzerinde herhangi bir mutajenik etki göstermemiştir.

Özet olarak, AVM 002 ve AVM 014, daha yüksek RT enzim inhibisyon etkisi ve düşürülmüş sitotoksik etki elde etmek üzere, bileşik yapısında küçük değişiklikler yapılarak geliştirilmek için en iyi özellikleri göstermiştir.

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ABBREVIATIONS

AIDS	:Acquired Immunodeficiency Syndrome
AMES	:Bruce N. AMES
BrDU	:Bromodeoxyuridine
CC_{50}	:50% cytotoxic concentration, or concentration required to reduce viability of the host cells by 50%
Con A	:Concanavalin A
DMSO	:Dimethyl Sulfoxide
EC_{50}	:50% effective concentration, or concentration required to inhibit HIV-1-induced cytopathicity by 50%
ELISA	:Enzyme-Linked Immonusorbent Assay
FBS	:Fetal Bovine Serum
FDA	:Food and Drug Administration
HIV-1	:Human Immunodeficiency Virus Type 1
IC_{50}	:50% inhibitory concentration, or concentration required to inhibit HIV-1 RT activity by 50%
LB	:Lysis Buffer
MTT	:3-{4,5-dimethylthiazol-2-yl}-2,5 diphenyl tetrazolium bromide
OD	:Optical density
PBMC	:Peripheral Blood Mononuclear Cells
PBS	:Phosphate-Buffered Saline
RPMI-1640	:Roswell Park Memorial Institude-1640
PHA	:Phytohemoglutine
RT	:Reverse transcriptase

Chapter 1

INTRODUCTION

Since the beginning of the history of humanity, epidemic diseases have been the most merciless killer. Plaque had been a horrible nightmare of the middle age people. Spanish flu killed nearly 22 million people around the world in 120 days of 1918 and today's modern world AIDS (Acquired Immunodeficiency Syndrome) has been the growing epidemic since June 5, 1981 in which Center for Disease Control and Prevention published morbidity and mortality generally seen in homosexual men. From then on AIDS spreads rapidly especially in sub-Saharan Africa and AIDS has become the most devastating disease human kind has ever faced. It is estimated that 40 million people were living with HIV at the end of 2001 and more than 60 million people have been infected with the virus (http://www.unaids.org).

AIDS (Acquired Immunodeficiency Syndrome) is the result of the replication of Human Immunodeficiecy Virus (HIV). Not all HIV infected person is called as an AIDS patient. HIV disease become AIDS when immune system of a body is so damaged, immunological abnormalities start to occur. That means having less than 200 CD4+ cell or opportunistic infections. It is the last period of HIV infections. These infections cause weight loss, brain tumors, and other health problems and also can kill infected person without treatment. Since the blood cells are very important for infected person, an ideal anti-HIV-1 chemotherapeutics should have desired properties. It is expected that the therapeutics should reach to desired beneficial effect with minimal adverse effects. While no chemicals can be certified as completely "safe" (free of risk) since every chemical is toxic at some level of dosage, it is possible to estimate the risk associated with exposure to the chemical under specified conditions if appropriate tests are performed.

Substantial amount of medicine against HIV-1 have being developed for the treatment of AIDS patience. Because eucaryotic cells do not have reverse transcriptase (RT) activity, RT has become one of the most important targets for the development of chemotherapic agents against HIV-1. RT shows both RNA and DNA dependent polymerase activity, and it converts viral RNA genome into double stranded proviral DNA that can integrate into host chromosome. A variety of RT inhibitors classified in nucleosid analogues (NRTIs) and non-nucleosid (NNRTIs) have been developed,

already. Today, there are two classes of RT inhibitors. These are nucleoside RT inihibitors (NRTIs) and non-nucleoside RT inhibitors (NNRTIs). All nucleoside analogues (NNRTI) can inhibit RT anzyme in a similar fashion, they work as a chain terminator but NNRTIs bind the hydrophobic pocket of HIV-1 RT. Because of the binding mode of NNRTIs, they are active only for HIV-1 RT enzyme not HIV-2 (De Clercq, 1998). However, each patient shows a different reaction to a specific RT inhibitor. In fact, some of the patients cannot tolerate many of the inhibitors. In addition, HIV-1 mutates and develops resistance against current drugs. Based on the information, it is necessary to continue RT inhibitor development in order to obtain new drugs with low toxic effect but maximum RT activity inhibition.

The aim of this study was to investigate the effects of nineteen benzaxsazole and bensimidasole derivatives compounds synthesized by Pharmacology Department of Ankara University on Reverse transcriptase enzyme inhibition. These compounds had been initially examined by the same research group in order to find their anti-bacterial effects but inhibitory effects of the compounds on RT were unknown. Therefore, quantitative determination of the enzyme inhibition by these compounds was achieved by non-radioactive reverse transcriptase assay in which incorporation of digoxigenin and biotin labeled dUTP into DNA by RT was measured as the indicator of enzyme activity. In addition, the compounds with RT inhibitory effect were further analyzed for their effects on viability, proliferation and activity of peripheral blood mononuclear cells in order to determine if these compounds are toxic on the cells. Finally, mutagenic effects of the compounds were examined on Salmonella typhimurium strains TA 100 and TA 102.

Chapter 2

HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 (HIV)

2.1. HIV-1

HIV is a lentivirus of the retrovirus Familiy and there are two types of HIV. The genomes of two types of HIV-viruses, HIV-1 and HIV-2, differ from each other by approximately 50-65 % on the nucleotide level. Originally HIV-1 strains were classified into different genetic subtypes by sequencing short genomic fragments, usually from the *env* and *gag* genes. Especially the *env* C2V3 gene region has been used frequently for subtyping analyses. When the envelope gene region is used, nine subtypes can be distinguished (A-H, J), but if the analysis is based on the *gag* gene, the E subtype strains cluster together with the A subtype strains. This has been interpreted such that these strains do not represent a single subtype but are A/E recombinants. True E subtype strains have not been found so far (Carr *et al*., 1996, Gao *et al*., 1996).

High-resolution electron microscopy of viral particle shows that virions have a spherical shape in about 110 nm diameter. Although the overall shape of virions is spherical, computer simulations of shadoved replicas and photomicrograps produced by scanning electron microscopy suggest that virions are icosodeltahedrons. Nucleocapsid is in 100 nm diameter and wide free end is in 40-60 nm, narrow end is in 20 nm width (Hirsc *et al*., 1996).

Infectious virus contains two single stranded RNA molecules about 9.2 kb long as a genomic material. After the entry of virion into the target cell, RNA is converted into DNA in the cytoplasm by reverse transcription. Precursor polypeptides for virion proteins and several additional open-read frames are encoded in RNA. There are three important genes; *gag, pol* and *env*. Encoded proteins by these three genes are cleavaged by host ezymes and viral enzymes. The *gag* gene encodes the precursor for virion capsid proteins MA, CA and NC, the *pol* gene encodes polymerase enzymes, and *env* encodes the precursor for envelope glycoproteins gp120 and gp41. The transcriptional transactivator (*tat*) and regulator of viral expression (*rev*) genes are each encoded by two overlapping exons and produce small nonvirion proteins which are essential for viral replication. There are also non-essential genes, designated ″accessory'' or

auxiliary genes, encoded by HIV-1. These are *vif, vpr, vpu and nef*. Products of these genes appear not to be packaged into virions (Hirsch et al., 1996).

As a group, the proviral genomes of primate lentiviruses display a high content of adenosine deoxyribonucleotide (A) residues (38% to 39%) and low frequency of cytidine deoxyribonucleotide (C) residues (16% to 16%). The generation of a A-rich genome may be due to an enzymatic property of RT and/or selective pressure during evalution of the virus group. For example the strong bias in the env gene for the triplet AAT and related codons favors serine, threonine, and asparagine. This bias appears to lead to the creation of new N-linked glycosylation sites, which could enable the virus to escape from the host immune response (Hirsch et al., 1996).

2.2. The Viral Enzymes

Viral enzymes are encoded by pol gene. Cleavage of the gag-pol polyprotein by protease enzyme yields PR, RT (and Rnase H) and IN (Hirsch et al., 1996).

2.2.1. Protease (PR)

Protease enzyme act as a zymogen and mature PR dimer is released by an autocatalytic cleavage possibly by a trans (intermolecular) mechanism. Active protease enzyme may be dimer because if protease enzyme is inactivated by site-specific mutations in pol gene, uncleaved gag and gag-pol polyproteins are released (Kohl *et al*., 1988, Loeb *et al*., 1989, Manchester *et al*., 1994).

In the dimer structure, N- and C-termini of both PR monomers are twisted together to form a four-stranded antiparallel β-sheet. The catalytic active site has specifity for more than one cleavage site. Upstream of the cleavage site is always hydrophobic and unbranched at the β-carbon. PR enzyme is inhibited by blocking enzyme dimerization with symetric inhibitors, stericomplementarity compouns of the active site (Kuo *et al*., 1994, Lam *et al*., 1994, Wlodawer *et al*., 1993).

2.2.2. Integrase (IN)

Linear double-stranded viral DNA converted from single stranded RNA by reverse transcriptase is inserted into the host cell genome covalently by viral IN enzyme. IN enzyme encoded by pol gene is proteolytically processed from Gag-Pol precursor polypeptide to yield 32 kd active enzyme (Goff, 1992).

The N terminus of HIV IN shows high sequence conservation that contains pairs of histidine and cysteine residues (His12, His16, Cys40, Cys43) that can be seen in some of DNA-binding proteins. Amino acid residues Asp64, Asp116, and Glu152 placed in a large central domain of IN are highly conserved in Ins of retroviruses and this central domain contains five-strand β-sheet and six helices like Rnase H domain of reverse transcriptase enzyme. Active integrase contains two monomers and dimerization process is thought to be essential not only for integration but also for assembly of these viral enzymes into virus particles. Although a molecule of IN contains a single catalytic site, these active sites seem to have separate binding sites for the ends of linear viral DNA and double-stranded host cell DNA. IN enzyme recognizes and binds the att sites of linear viral DNA and then cuts off a deoxythymidylate (TT) dinucleotide from the 3′ ends of double-stranded viral DNA and also host DNA to produce a staggered 5–6 overhang. 3′ end in the substrate DNA is joined to the 5′ phosphoryl end in target DNA (Bushman *et al*., 1993).

2.2.3. Reverse Transcriptase (RT)

Reverse transcriptase, a key enzme in viral life cycle, is an RNA-dependent DNA polymerse. It converts the single stranded viral RNA molecule into a double stranded proviral DNA molecule. Before the discovery of HIV infection, RT enzyme was discovered in 1970 by David Baltimore and Howard Temin. Since RT is essential for viral replication, it becomes an important target for drugs against AIDS. Enzyme consists of two subunits; p51 and p66. Gag-pol polyprotein ($Pr160^{gag\text{-}pol}$) is cleaved by PR enzyme to yield p66, which form a homodimer. Subsequently one subunit of p66 in the homodimer is cleaved by PR enzyme near the C-terminus. Occuring heterodimer contains two identical units but one of them does not contain RNase H subdomain due to the cleavage (Figure 2.1) (Skalka *et al*., 1993, Hirsch *et al*., 1996).

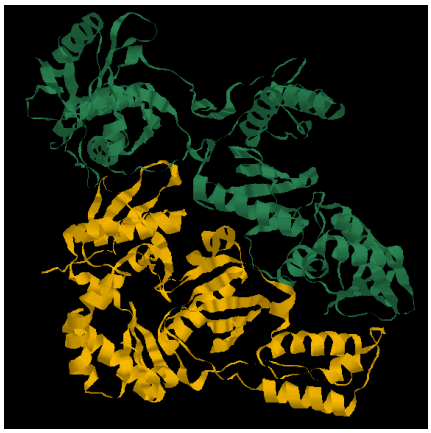


Figure 2.1. RT enzme p66 is in green colour, p51 is in gold in colour (http://chemistry.gsu.edu/CAISER/modules/rt/reverse_transcriptaseh.html)

The active form of HIV-1 RT enzyme is produced in a two-step dimerization process. First step of dimerization process is the rapid association of the two subunits, and second is following conformational change that gives fully active form (Divita *et al*., 1995, Divita *et al.*, 1995a). The amino acid residues that participate in subunit interactios are found in the connection and thumb subdomain of the p51 subunit and in the connection and palm subdomains of the p66 subunit, and to a lesser extend, in the tip of the thumb subdomain of the p51 subunit and the RNase H region of the p66 subunit. First step includes interaction of the tryptophan cluster at the interface of the connection subdomain of two subunits. Two important triptophan residues 389 to 422 form hydrophobic patch in the region containing α-helix L and β-strand 19 (Wang *et al*., 1994, Becerra *et al*., 1991, Baillon *et al*., 1991). It was found that the N-terminal sequence of the Trp cluster is the most efficient in association and dimerization process. Conformational change stacks the thumb subdomain of p66 and places the fingers subdomain of p51 in the palm subdomain of p66 (Divita *et al*., 1995). Association and activation rate of two subunits have been found to be $5{,}1 \times 10^4$ M^{-1} s^{-1} (May *et al*., 1999) and heterodimer can be formed 10-10^3 times faster than the p66 homodimer for motion (Becerra *et al*., 1991). Because of the importance of the Trp clusture in formation of active RT, synthetic peptides targeting the dimerization process can be used as an anti-HIV-1 therapeutics. Heterodimers of RT interact in a head-to-tail configuration.

The p66 subunit contains five subdomains; fingers region, palm region, thumb region, connector region, RNase H region.

Four subdomains in the p66 subunit of the heterodimer are arranged side-by side to yield an elongated RT domain and a connection subdomain in p66 join the RT and RNase domain. In this model, the structure of RT looks like a right-hand. The catalytic site of RT lies in a cleft in the palm of the p66 subunit and contains the sequence Tyr83-Met184-Asp185-Asp186. These amino acids are highly conserved not only in retroviral RT, but also in other DNA polymerases. In addition, Asp110 also participates in catalytic activity (Figure 2.2) (Fields, *et al*., 1995).

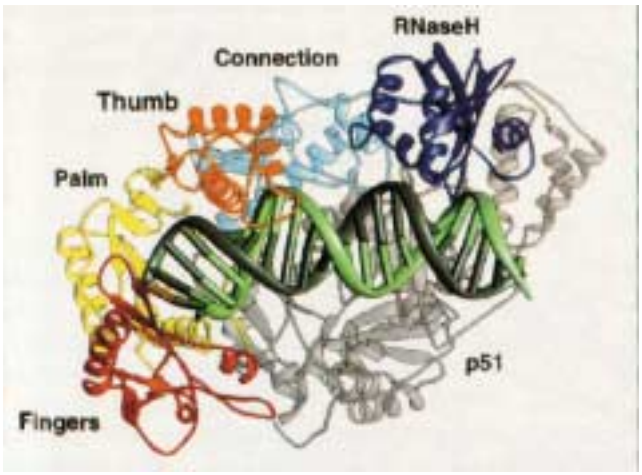


Figure 2.2. RT subdomains and catalytic active sites shown in white colour, Fingers region (red) Residues 1-90 and 111-160, Palm region (yellow) Residues 91-110 and 161-240, Thumb region (orange) Residues 241-310, Connector region (cyan) residues 311-430, Rnase H region (purple) Residues 431-end. (http://chemistry.gsu.edu/CAISER/modules/rt/reverse_transcriptaseh.html)

After binding of free nucleotides, fingers and thumb subdomains show large conformational changes. The finger domain closes down on the palm domain, trapping the template strand and the nucleotide. It is assumed that fingers then turn back to the open position, releasing the molecule of pyrophosphate leftover from the nucleotide, allowing reverse transcriptase to take up the next nucleotide (Fields *et al*., 1995). These are the first two conformational changes. Third change is assumed to position the enzyme at the new 3′ end of the growing DNA chain.

In this mechanism, while the finger domain (residues 74-78, 89 and 151-152) holds the template strand in place, the palm subdomain (residues 110-117, 160-161, 183-186, and 219-221) positions the nucleotide being incorporated.

Close positioning of 3′-hydroxyl terminus of primer to the catalytically essential Asp110-Asp185-Asp186 residues results in a nucleophilic attack on the α-phosphate of an incoming nucleoside triphosphate. Three aspartic acid side chains, oxygen on the α-phosphate of the nucleotide and possibly with a water molecule produces an octahedral shell. Positioning of α-phosphate of nucleotide in this shell gives change for an in-line attack by the 3′ hydroxyl group of the complementary strand. The metal ions assist the catalysis by juxtaposing the reactive primer hydroxyl with the entropic energy of nucleophilic attack. RT incorporates twenty nucleotides into DNA per second (Fields *et al*., 1995). The Km value of RT for dTTP as substrate and poly(rA)oligo$(dT)_{12\text{-}18}$ as a primer, derived from Eadie-Hostee plots is 4,6±0,1 μM and k_{cat} value is 1,03± 0,02 s^{-1} (Reardon *et al*., 1995). Hence reverse transcription process occurs in the cytoplasm of the infected cell, some reverse transcription seems to be initiated before or during assembly of the virion.

Connection subdomain of HIV-1 RT interacts with template-primer also links the RT and RNase H domain. RNase H domain contains two divalent metal ions and 3′ end of the template strand contacts the active site of RNase H domain. Backbone phosphates of the template interact with these metal ions (Restle *et al*., 1990). Interactions between the two connector regions and between thumb on the p51 subunit and RNase H domain on the p66 stabilize the heterodimer form of HIV-1 RT. While viral DNA is synthesized, RNase H subdomain degrades viral RNA at the 3′ position, 16 to 18 nucleotides away from the site of synthesis.

Activity of reverse transcriptase is a key factor to explain viral diversity. All RTs lack the 3′→ 5′ exonuclease activity necessary for proof-reading during DNA synthesis. In other words, extremely tolerant of non-standart base pairs and modified ribose units. The misincorporation rate is about 1/1700 to 1/4000 bases, but some bases in genome misincorporation rate increases to 1/30 bases. It means in each replicative cycle 10 bases change in HIV-1 genome (Lori *et al*., 1992). This high mutation rate can explain how virus can escape from immune responses. It can also generate resistancy for anti-HIV chemotherapeutics. In addition to direct misincorporation, slippage of the two DNA strands at repetitive sequences can cause a deletion (unpaired nucleotide(s) in

template strand) or addition (unpaired nucleotide(s) in the primer strand). Moreover, frameshifts are caused by misincorporation followed by misalignment of the template-primer. The most common type of mutations observed *in vivo* is point mutations. These are simply caused by the incorporation of a wrong nucleotide to the growing chain. A tpical feature of a lentiviral RT is a bias to induce G→A mutations, which creates hypermutant genomes (Vartanian, *et al*., 1991). This might contribute to the high A content of lentivirus genomes.

Each of these mechanisms can yield single nucleotide mutations and may also operate over large distances to produce changes involving many nucleotides.

2.3. Life Cycle of HIV-1

Viral life cycle can be divided into two phases; early and late phases. Binding of gp 120 to the CD4 receptor starts viral infection. After attachment, gp 41 mediates the insertion of nucleocapsid into the cytoplasm where uncoating and reverse transcription events occur. Synthesized double stranded proviral DNA is then transported to nucleus and inserted into host DNA. This ends the early phase of viral life cycle. In the late phase, transcription and processing of viral RNA occure. Unsipliced, singly spliced and multiply spliced mRNAs are transported to the cytoplasm and processed to yeald viral proteins. Full-length viral transcripts interacts with virion precursor polypeptides and accumulate under the host cell plasma membrane to produce immature nucleocapsid.

2.3.1. Virion Attachment and Entry

Interaction between the extracellular domain (gp 120) of the env and CD4 antigen give rise to change in conformation of Env gp 41 to produce fusion active site (Vartanian *et al*., 1991,David *et al*., 1998). D1 immunoglobulin like domain of CD4 contains a high-affinity binding site for gp 120. Cytoplasmic transmembrane C-terminus portion of CD4 is noncovalently associated with src-related tyrosine kinase $p56^{lck}$ that is thought to mediate signal transduction. However $p56^{lck}$ is thought to start signal transductoion, studies with HeLa cells contains deletion mutations in CD4 cytoplasmic tail shows that extracellular domain of CD4 is enough for virus fusion, infectivity and syncytium formation, and signal transduction is not neccessary for infectivity (Diomond *et al*., 1990).

Interaction of gp 120 with CD4 receptor on cell enhances exposure of the V3 loop. Binding mediated conformational change or presumed way that cleavage of V3

loop by cellular protease in plasma membrane (Murakami *et al*., 1991) after attachment leads to dissociation of the gp 120 subunit from the gp 41 subunit in virion membranes (Kido *et al*., 1990). In addition cellular protein disulfide isomerase, localized in plasma membrane is thought to reduce critical disulfide bonds in the env gp after virions attach to the CD4 receptor. Inhibition of this enzyme results in blocking the viral infectivity and presumably leaves gp 120 in unchanged conformation. Change in conformation of Env gp is thought that gp 41 subunits within gp 120 oligomers form stable coiled coils and this event produces a fusion domain that is inserted into the cell plasma mebrane. In this process important role belongs to hydrophobic fusion peptide at the N-terminus of gp 41 (amino acid residues 517-527) (Morikawa *et al*., 1991). Leucine zipper (amino acid between Leu 553 and Leu 590) also has a role in fusion (Wild *et al*., 1994). Condensation of receptor is thought to bring cell mebrane and virus membrane in close position to fuse each other (Moore *et al*., 1990).

CD4 receptor is necessary but not enough to mediate HIV infection. There must be an additional (receptor chemokine) receptor on target cell membrane. Chemokines are cell surface membrane-bound fusion mediating molecules. The chemokine coreceptors are the CXC family (CXCR1 to CXCR5) and the CC family (CCR1 to CCR9). Conformational change occurred after gp 120 binding on CD4 is not sufficient for fusion. Conformational change produced in the gp 41 by the chemokine receptor is the complementary event of membrane fusion of HIV-1 and host cell (White, 1992). Selective infection of HIV-1 strains is also explained by differences in chemokine coreceptors found in a target cell. When chemokine receptors are accounted, HIV-1 can be classified into three main groups. They are T-tropic SI strains that selectively interact with the CXCR4 chemokine receptor found on lymphocytes, M-tropic NSI strains that interact with the CCR5 chemokine coreceptor and, CCR2 and CCR3 found on macrophage. Dual tropic HIV-1 strains can use more than one chemokine coreceptor. Lately a cofactor CCR8 has been identified that allow infection of either by T-cell tropic or by M- tropic strains of HIV. Mutations in chemokine receptors can help to understand how resistancy in some cases is seen but mutations in HIV-1 may accomplish this phenomenon (Murdoch *et al*., 2000).

2.3.2. Reverse Transcription

Reverse transcription of viral single stranded RNA molecule into double stranded DNA molecule is started at primer binding site (PBS) from 3′ CCA end of the

$tRNA^{lys}$ that bind PBS by hydrogen bonds. In this process RT makes two jump or strand transfer. First strand trasfer occurs when RT comes to a strong stop sequence at short repeat (R) of 5′ end of viral RNA genome (Montano *et al*., 1997, De Stefano *et al*., 1991). Pausing of the enzyme during synthesis promotes strand transfer (Schatz *et al*., 1990, DeStefano *et al*., 1991). RNase H activity, subsequently, degrades RNA molecule. Synthesized R sequence of (-) strand DNA is complementary to 3′ R sequence of viral RNA. First jump to the 3′ gives change to match both R sequence and to continue (-) strand elongation on the viral RNA template through U3 and into the viral genome. RNase activity leaves polypurine tract (PPT) at 3′ sequence of 3′ LTR. PPT serves as a start point for (+) strand DNA. Second jump is neccessary to continue (+) strand DNA synthesis and it occurs through 3′ PBS. Base pairing between two PBSs is formed and (+) strand completion gives full-length double stranded proviral DNA that is ready to be integrated into the host cell genome (Delwart *et al*., 1990, Buiser *et al*., 1993).

2.3.3. Integration of Proviral DNA Trancription of Viral mRNAs

Translocation of preintegration complex into nucleus is an active transport process. It is mediated by nuclear localization signals in MA or Vpr. Integration is not random (Peliska *et al*., 1992, Varmus *et al*., 1989). HIV-1 genome is generally inserted into or near two repeated DNA elements in the human genome: the L1 and Alu elements (Shin *et al*., 1988). These are also called as retroposons that may make chromatin structure attractive for integration. IN generally prefers DNA segments having a wide major groove or pre-existing deformations of DNA within the nucleosome core for integration (Stevens *et al*., 1994, Preston *et al*., 1988).

A protein, integrase interactor 1 (Ini 1) was found to bind to HIV IN and stimulate its DNA joining activity *in vitro*.

Integration of proviral DNA into host genome leaves single-stranded gaps and two mismatched nucleotides at each 5′ terminus. Host cell enzymes remove the mismatched nucleotides, fill in the gaps, and nick seal the remaining ends.

Control of viral transcripts level is mediated by tat and rev viral proteins. U3, R and U5 domains in LTR show different functions in viral gene expression. The U3 domain contains TATAA box as an initiation site of host RNA polymerase II, and sites for binding the cellular transcription factor SP1. Initiation of the viral transcripts occurs

at the U3/R border of the LTR sequence. In R sequence tat binding site tat-response element (TAR) is located. Rev controls the ratio of spliced to unspliced viral mRNA and functions through a Rev-response element (RRE) in the full-lenght transcripts. Rev gene product also mediates transport of viral RNA from the nucleus to the cytoplasm. Synthesized viral mRNAs are transported to the cytoplasm and translated into proteins by host cell mechanisms. Full-length messenger RNA (mRNA) transcripts are produced from the provirus and are used for translation of Gag and Gag-Pol polyproteins, or they can be spliced into smaller mRNAs that are used to produce Env proteins and the non-structural proteins. Full-length transcripts are also used as genomic RNA that is packaged into new progeny virions (Pryciak *et al*., 1992).

Cellular cytokines such as tumor necrosis factor a (TNF-a) can activate quiescent T cells (and at the same time it also activates HIV-1 replication) by inducing nuclear factor kB (NFkB) in the host cell, which in turn binds to specific enhancer motifs in DNA and activates transcription (Tscherning *et al*., 1998). HIV-1 has two NFkB binding sites in the U3 region of the LTR, while HIV-2 and SIV only have one site. In addition, HIV-1 subtype C has been reported to possess three NFkB sites instead of the normal two and it has been speculated that it might influence the higher pathogenicity of this subtype (Nabel 1997, Johansson *et al*., 1995, Gao *et al*., 1996b, Salminen *et al*., 1996). Also the A/E strains prevalent in Southeast Asia have been found to contain one functional NFkB site and one variant site with reduced activity (Montano *et al*., 1997)

2.3.4. Virion Assambly and Maturation

Interaction of gag polyprotein $Pr55^{gag}$ (Geldable, 1991, Vaishnav *et al*., 1991) produces an assembly intermediate in the cell cytosol. Nucleoprotein complex contains $Pro55^{gag}$ and gag-pol polyprotein $Pr160^{gag\text{-}pol}$ and genomic viral RNA. Packaging element (psi) located at the 5′ end of full-length viral RNA is recognized by NC domain in the gag polyprotein. After the insertion of Env gp oligomers into host cell membrane, MA domain of $Pr\ 55^{gag}$ interacts with the cytoplasmic tail of the TM subdomain in the Env oligomer. Budding of the viral nucleoprotein complex through the plasma membrane gives immature viral particles. Gag, Gag-Pol and Env gp procursors are processed to reach infective mature viral particles (Kohl *et al*., 1988).

2.4. RT Inhibitors

Finding of suramin to protect human T lymphocytes against the infectivity and cytopathicity of HIV has started the anti-HIV chemotherapy (Wills *et al*., 1991). Shortly after, successful isolation of HIV in cell culture (Mitsuya *et al*., 1984, Barre-Sinoussi *et al*., 1983), inhibitory effects of suramin and polyoxometalate HPA 23 (Popovic *et al*., 1984) on RT was shown. But suramin was discontinued as a therapeutic modality for the treatment of HIV infections because additional short-term clinical studies showed that it is too toxic and of little, if any, clinical benefit (Rozenbaum *et al*., 1985).

The replicative cycle of HIV gives excellent targets for chemotherapeutical aims. These targets are;

i. Viral adsorption to the cell membrane
ii. Fusion between the viral envelope and the cell membrane
iii. Uncoating of the viral nucleocapsid
iv. Reverse transcription of the viral RNA to proviral DNA
v. Integration of the proviral DNA to the cellular genome
vi. DNA replication
vii. Transcription of the proviral DNA to RNA
viii. Translation of the viral precursor mRNA to mature mRNA
ix. Maturation of the viral precursor proteins by proteolysis, myristylation, and glycosylation
x. Budding, virion assembly and release

Since RT is essential for virus replication and has no closely related identified cellular homolog, it has been the prime target for antiviral therapy against the AIDS (Kaplan *et al*., 1987). After discovery of AZT (3′-azido-2′, 3′-dideoxythymidine) to inhibit reverse transcription selectively both *in vitro* and *in vivo*, the search for clinicaly useful drugs for treatment of AIDS was initially focussed on inhibition of HIV reverse transcriptase enzyme (De Clerck, 1992). Together with nucleosid analogues there are two more classes of inhibitors approved by FDA for chemotherapy of AIDS. Other two classes are non-nucleosideinhibitors of RT and protease inhibitors.

2.4.1. Nucleoside Analogues

Nucleoside analogue inhibitors (NRTIs) of RT enzyme of HIV-1 were the first class of compounds to be used in anti-HIV-1 therapy. The first approved drug AZT (zidovudine) belongs to the 2′,3′-dideoxy class of nucleoside analogues. Other members of this class of compounds are d4T (stavudine), ddI (didanosine), ddC (zalcitabine), the heterosubstituted (-)- enantiomer of 2′-deoxy-3′-thiacytidine, 3TC, and the carbocyclic analogue abacavir (1592U84) (Duluge *et al*., 1997). All approved nucleoside analogue inhibitors of RT are 2′,3′-dideoxyl derivatives of the natural nucleotide substrates of DNA polymerases. They show their inhibitory effect in the same way (Figure 2.3). It can be said that all nucleoside analogues can be inhibitors of RT but small modifications of chemical structure can change their properties such as their inhibitory activity and toxic effects on the organism.

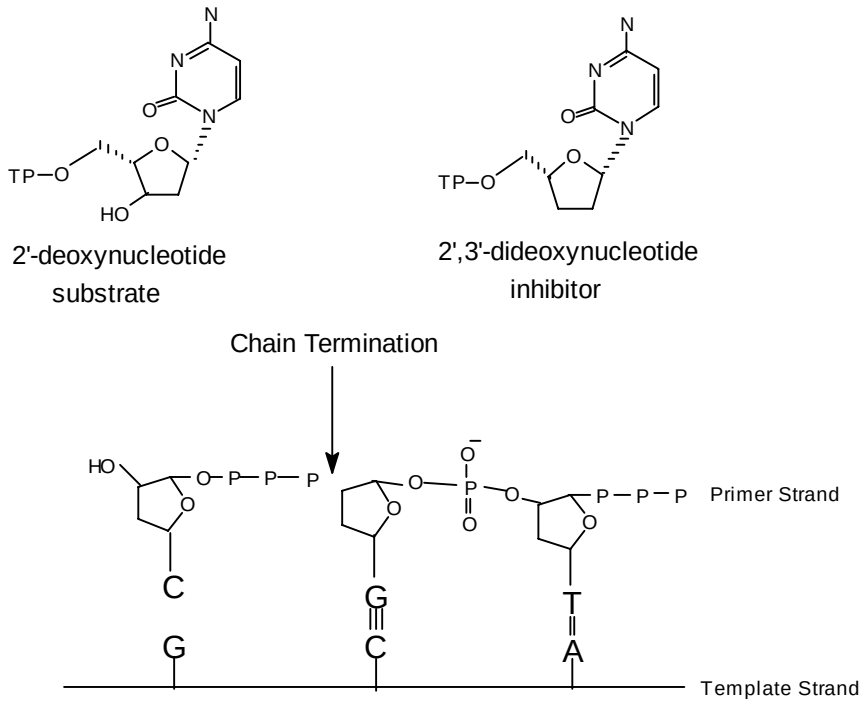


Figure 2.3. Chain termination by NRTI

First step (Figure2.4) is the formation of the monophosphate (Step A) second step**,** the formation of diphosphate (Step B) by nucleotide kinases is followed by

formation of triphosphate (Step C) results from the action of nucleoside kinases or other less specific phosphorylation enzymes.

(http://www.bristol.com.br/mediços/infocientifica/6/default.htm)

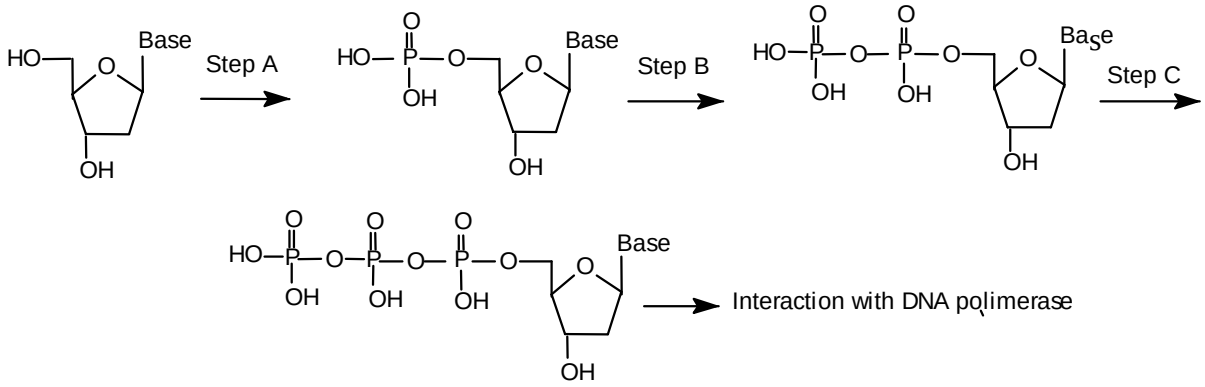


Figure 2.4. Phosphorylation of natural D-2′-dideoxynucleotides

First thymine analogue AZT is converted into monophosphate form easily but 3′ N_3 group of this compound causes trouble in work of enzyme in Step B. This phenomenon shows that activity of nucleosid analogues depend on the substrate activity of kinase enzymes. Zerit® (stavudine, d4T) was synthesized to deal with this problem by modifiying chemical structure of AZT. Change in 3′ N_3 group of AZT to H molecule improved mono-, di-, triphosphate ratio from 8:1:1 to 1:1:1 (Schmit & Weber, 1998).

Another nucleoside analogue Abacavir is phosphorylated by different process. Intracellularly monophosphate formation is followed by deamination to (-)-corbavir triphosphate with catalysis of adenosine deaminase or adenylicasid deaminase.

Parameter that defines the activity of kinase enzyme is enantioselectivity of the enzyme to optic isomers of sugar molecule. Two main carbohydrates found in nucleic acids, ribose and deoxyribose are naturally in D-configuration and studies initialy focused on analogues in D-conformation. Moidity of analogues that have L-sugar is restricted by enantioselectivity of kinase enzymes especially act in step A (Figure 2.5) (Zemlicka, 2000).

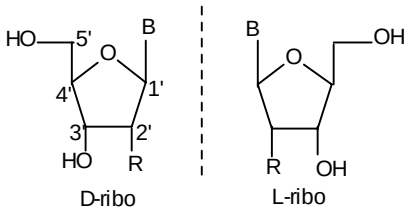


Figure 2.5. Structure of D- and L-nucleosides

Studies revealed that, stavudine, zidovudine and didanosine show very strict D-form selectivity. L enantiomer of AZT shows 25,000-fold decline in RT inhibition activity. On the other hand, L-enantiomers of nucleoside analogues show limited cytotoxicity lower than D forms (Zemlicka, 2000).

Advers events seen with usage of NRTIs are bone marrow supression, anemia or neuropenia, GI intolerance, headache, insomnia, asthenis, pancreatitis, peripheral neuropathy, nausea, dierrhea, stomatitis, hypersensitivity reaction and most important lactic asisdosis. It is thought that these complications are the results of the toxic effects on mitochondria. Competition of analogues with the substrate of mitochondrial polymerase-g (mDNA) results in decline in 13 important mitochondrial protein (http://www hivinsite .ucsf. edu).

2.4.2. Nonnucleoside RT Inhibitors

Discovery of 1-(2-hydroxyethoxymethyl)-6-(phenylthio)thymine (HEPT) (Baba *et al*., 1989, Miyasaka *et al*., 1989) and tetrahydroimidazo[4, 5, 1-jkj][1, 4] benzodiazepin-2(1H)-one and –thione (TIBO) (Pauvels *et al*., 1990, Debyser *et al.*, 1991) has been showed to inhibit HIV-1 RT specifically, research has been focused on NNRTIs. It was followed by the discovery of nevirapine (De Clercq, 1993) (diriyridodiazepinones), pyrididone (L-697,661), BHAP (delavirdine, U-90152), and other HEPT series compounds (i.e. I-EBU(MKC-442), TIBO series [(8-ckloro-TIBO(tivirapine, R 86183)] and PETT (trovirdine, LY 300 046). There are now over 30 compounds that are defined as a specific RT inhibitors (De Cleccq, 1994).

Trovirdine is the first generation of PETT (phenylethylthiazolythiourea) series. Urea analogues obtained from further optimization of PETT series was found to have less toxicity than thiyourea compounds (Högberg *et al*., 1999).

Nevirapine, delavirdine and efavirenz were approved in all these compounds to be used on AIDS patients (De Cleccq, 1994).

NNRTIs interact non-competitively with a specific pocket site of HIV-1 RT. This pocket is closely associated with, but distinct from the NRTI binding site. The distance between two binding site is about 10 Å (Tantillo *et al*., 1994) and works allostericly. The cooperative interaction between these two sites (Spence *et al*., 1995) provides higher binding activity of NNRTIs to enzyme-substrate complex (Althans *et al*., 1994) than the free enzymes. Cooperative interaction between these two sites increases effectiveness of NRTI and NNRTI combination therapy, because binding of NNRTI to their hydrophobic pocket decrease the binding rate of substrate into growing DNA. NNRTIs are only effective for HIV-1 RT because of the presence of highly hydrophobic flexible pocket that is not seen in any RTs or DNA polymerases (Kohlstaedt *et al*., 1992, Nanni *et al*., 1993). Important amino acids that participate in the binding of NNRTIs have been determined by drug resistancy mutations. Leu100, Lys103, Val106, Val108, Glu138, Val179, Tyr181, Tyr188, Gly190, Met230, and Pro236 were determined as important amino acid residues for inhibitor interaction with HIV-1 RT. Except Gly138 localized in p51 subunit, all of these amino acids are localized in p66 subunit (De Clerck 1999).

Commonly seen binding mode of NNRTIs to their binding site cause a repositioning of the three-stranded β-sheet in the p66 subunit containing the catalytic aspartic acid residues 110, 185, and 186. This conformational change turns active site into inactive. α-APA R95 846, 8-chloro-TIBO (R86183) (Ding *et al*., 1995), 9-chloro-TIBO [R82913] (Ren et al., 1995) and nevirapine binding to hydrophobic pocket of RT cause to change conformation into butterfly-like shape. It is suggested that these inhibitors act as π-electron donors to aromatic side-chain residues surrounding the pocket (Kroger, *et al*., 1995).

When delavirdine binds into the pocket, H-bonding to the main chain of Lys103 and extensive hydrophobic contacts with Pro236 stabilize the complex. Binding of U-90152 creates a channel between Pro236 and the polypeptide segment 225-226 and 150-106. The thiocarboxyanilides bind the pocket through hydrogen bonding with the main chain oxygen of Lys101 and hydrophobic interactions with Leu100, Val106, Val179, Tyr188, Phe227, Leu234 and His235, also Trp229 participate hydrophobic binding when thiocarboxanilide UC-781 interacts with pocket (Esnoulf *et al*., 1997, Plemans *et al*., 1998).

Unique specificity of NNRTIs makes them more useful than NRTIs such as they can inhibite RT in nanomolar concentrations that are at least 100,000 fold lower than their toxic concentrations. In addition they do not need phosphorylation process that restricts NNRTIs activity and they do not bind other polymerase enzymes (De Clercq, 1995).

Drugs used for anti-HIV-1 chemotherapy target the polymerase activity of RT but their toxic effects and emergency of drug resistant strains are the forces to develope new drug and strategies. DPC 961 with IC_{50}= 0,36 nM and DPC 083 with IC_{50}= 0,4 nM were developed for the patients that shıw multi-resistancy to nevirapine, delavirdine and efavirenz. DPC 961 is the second generation of efavirenz and developed by change in benzoxazine ring into quinaxaline (Bacheler, XIII International AIDS Conference Abstracts).

2.4.3. Recent Studies on Newly Synthesized Benzoxazole, Benzimidazole, Benzothiazole Derivatives

Several substituted benzimidazole derivatives have been reported for their antimicrobial, antitumor and antiviral activities after thiabendazole (2-[4-thiazolyl] benzimidazole) have been found to be effective in the treatment of helmintic diseases with blood clinical efficiency. Before that, 2-(α-hydroxybenz)benzimidazole (HBB) and 1,2-bis(2-benzimidazolyl)-1,2-ethanediol derivatives were introduced as an inhibitor of poliovirus replication (Ören *et al*., 1997).

A series of substituted 2-phenylbenzimidazole-4-carboxamides were synthesized and eveluated for their antitumoral activity *in vitro* and *in vivo*. They were found less toxic but some of them had antileucemic effects. In addition, substituted pyrimido[1,6-a]benzimidazoles were exhibited as a new potent DNA gyrase inhibitors. Recently benzoxazoles that are the isoesters of the benzimidazole derivatives, point out potential antimicrobial activity with lower toxicity. An antibiotic calcimycin (A 23187) includes benzoxazole ring in molecular stracture and weas found to be very effective for gram (+) bacteria. Moreover, series of 2-(4-aminophenyl) benzothiazoles substituted in the phenyl ring were defined as potent antitumor agents for breast cancer cell lines. 2-(4-amino-3-methylphenyl)-benzothiazole derivative was found as the most potent compound in these series as its activity extends to ovarian, lung and renal cell lines (Shi et al., 1996).

In addition to these observations both 3-([benzoxazol-2-yl]ethyl-5-ethyl-6-methylpyridin-2-(1-H)-one (L-696,299) and 3-(4,7,dichlorobenzoxazol-2-yl methylamino-5-ethyl-6-methyl-pyridine-2(1H)one, (L-697,661) are benzoxazole derivatives and are highly selective inhibitors of HIV-1 RT (Shi *et al*., 1996).

Studies on Topoisomerase I revealed that substituted benzimidazoles were inhibiting this enzyme with minimum or no DNA binding affinity, also substituted pyrimido[1, b-a] benzimidazoles were found to be a new class of potent DNA gyrase inhibitors (Hubschwerler *et al*., 1992).

Yalçın *et al*. 1990, 1992 reported antimicrobial activities of various 2,5-disubstituted benzoxazoles, benzimidazoles, benzothiazoles and oxazolo (4, 5-6) pyridines against some gram positive and negative bacteria and the yeast *Candida albicans*. Sicnificant antimicrobial activity was observed on enterobacter *Pseudomonas aeruginosa* and the yeast *C. albicans*. For *Klebsiella pneumoniae and C. albicans*, oxazolo (4,5-6)pyridine derivatives were observed to have the best inhibitory potency and antimicrobial activity (Ören *et al*., 1997, Yalcın *et al*., 1990, Yalcın *et al*., 1992).

Substituted benzimidazoles, benzoxazoles and related heterocycles, owing fused heterocyclic nuclei, are the structural isoesters of nucleotides. These constractions give them the chance to interact easily with biopolymers, and decreasing cytotoxic effects and increasing chemotherapeutic moidety (Hubschwerlen *et al*., 1992, Perrin *et al*., 1996, Kim *et al*., 1996, Shi *et al*., 1996).

2.4.4. The Drug Discovery, Development and Approval Process

The drug discovery, development and approval process take 15 years on average. Among the 5000 compounds tested through preclinical testing, only 5 of them were approved to be used on human testing and only one of these five is approved for sale.

In perclinical tests, compounds are tested in the laboratory to answer if the compound shows activity against target disease or not, and evaluate for safety. First step of this process is performed by using cells that can be special cell lines or blood cells directly taken from patients or healthy volunteers then animal tests are applied. Preclinical tests are followed by clinical trails to determine safety and dosage in healthy volunteer. After side effect determination in Phase II and Phase III clinical trails, data of compounds are analyzed by FDA (Food and Drug Administration).

In order to achive the aim of this study, first stage of preclinical tests was done. For this purpose, the inhibitory effects of newly synthesized compounds AVM 001, AVM 002, AVM 003, AVM 004, AVM 005, AVM 006, AVM 007, AVM 008, AVM 009, AVM 010, AVM 011, AVM 012, AVM 013, AVM 014, AVM 015, AVM 016, AVM 00,17, AVM 018 and AVM 019 (structures were given in Material Method section) on RT enzyme of HIV-1 were determined wether compounds show activity to target or not. Subsequently, in order to elucidate the effects on biological system, short-term cytotoxicity assays were performed and completed as given in the Drug Discovery, Development and Approval Process Table 2.1.

	Discovery/ preclinical testing	Clinical trials			FDA		Phase IV
		Phase I	Phase II	Phase III			
Years	**6,5**	**1,5**	**2**	**3,5**	**1,5**	**15 total**	
Test population	Laboratory and animal studies	20 to100 healthy volunteers	100 to 500 patient volunteers	1,000 to 5,000 patient volunteers	Review process/ approval		Additional post-marketing testing required by FDA
Purpose	Assess safety, biological activity and formulations	Determine safety and dosage	Evaluate efectiveness, look for side effects	Confirm effectiveness, monitor adverse reactions from long term use			
Success Rate	5,000 compunds evaluated	5 Enter trials			1 approved		

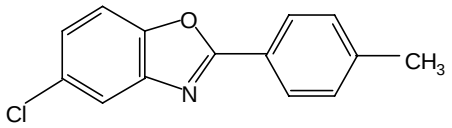
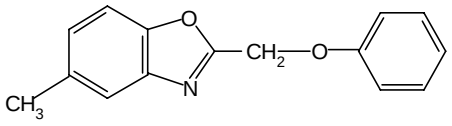
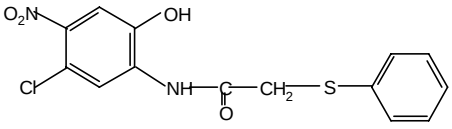
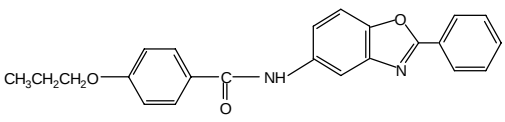
Table 2.1. Drug discovery, development and approval process

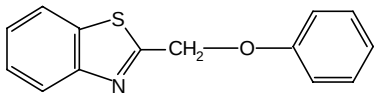
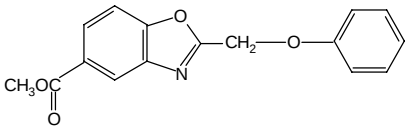
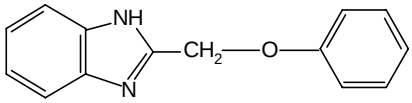
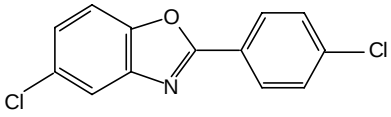
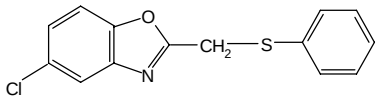
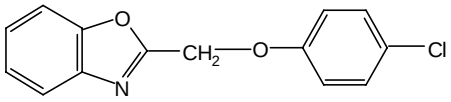
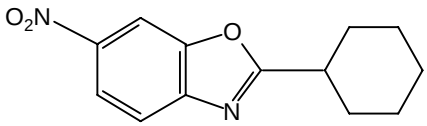
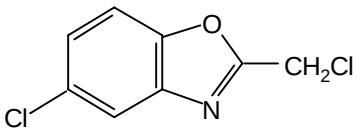
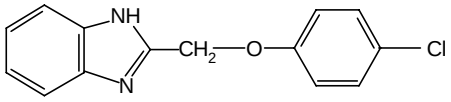
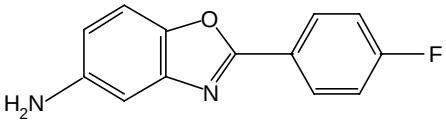
Chapter 3

Materials and Methods

3.1. Materials

Reverse Transcriptase Assay, colorimetric kit and heparin-sodium (Lequimine) were purchased from Roche. Cell Proliferation assay kit was purchased from Amersham Life Science. Roswell Park Memorial Institute (RPMI)-1640 medium, Fetal Bovine Serum (FBS), gentamycin sulfate, concanavilin A (con A), trypan blue, MTT, DMSO, luria agar were obtained from Sigma Chemical Company. Biocoll cell separation solution (Ficoll with desity 1,071 mg/ml) was from Biochrom Chemical Company. Potassium monohydrogen phosphate, potassium dihydrogen phosphate, sodium chloride were purchased from Merck Chemical Co. Tissue culture plates were from Corning Star, polyprophylene tubes and disposable pipettes were obtained from Greiner. Calcium phosphate (tri basic) was purchased from Aldrich Chemical Co. *Salmonella typhimurium* strains were obtained from Prof. Bruce Ames (Biochemistry Depertment, University of California, Berkeley). Newly synthesized compounds were kindly provided by Faculty of Pharmacy, Ankara University.

Compaund Code	Chemical name	Chemical Structure
AVM 001	5-Chloro-2-(p-methyl) phenylbenzoxazole	
AVM 002	5-Methyl-2-(phenoxymethyl)benzoxazole	
AVM 003	N-(2-hydroxy-4-nytro-5-chlorophenyl)phenylthioacetamide	
AVM 004	2-phenyl-5[(p-propoxyphenyl) carbonilamino]benzaxzole	

AVM 005	2-(phenoxymethyl)benzotiazole	
AVM 006	Methyl ester of 2-phenoxymethyl-benzoxazole-5-carboxylic acid	
AVM 007	2-(phenoxy)benzymidazole	
AVM 008	5-chloro-2-(p-chlorophenyl) benzoxazole	
AVM 009	5-chloro-2-(phenyltiomethyl) benzoxazole	
AVM 010	2-(p-chlorophenoxymethyl) benzaxozole	
AVM 011	6-nytro-2-cyclohexyl benzoxazole	
AVM 012	5-chloro-2-(chloromethyl) benzoxazole	
AVM 013	2-(p-chlorophenoxymethyl) benzimidazole	
AVM 014	2-(p-fluorophenyl)-5-aminobenzoxazole	

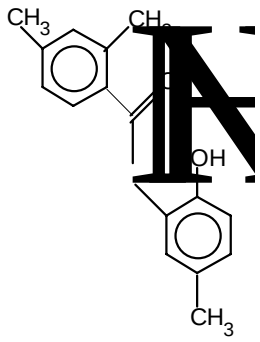
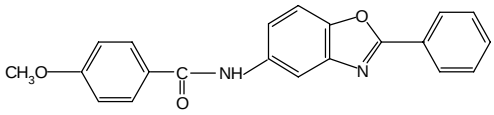
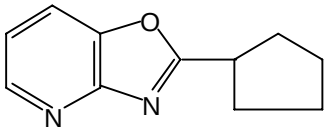
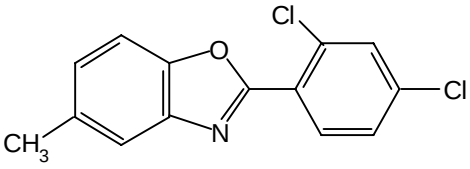
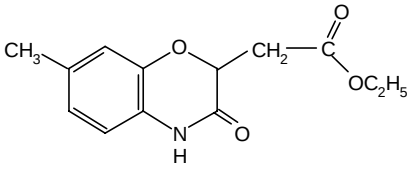
AVM 015	N-[(2-hidroxy-5-methyl)phenyl]-2,4-dimethyl-bensamide	
AVM 016	2-phenyl-5-[(p-methoxyphenyl) carbonylamino]benzoxazole	
AVM 017	2-cyclopentano-oxozalo(4.5-b) pyridine	
AVM 018	5-methyl-2-(2^1,4^1-dichlorophenyl) benzoxazole	
AVM 019	Ethyl 3,4-dihydro-7-methyl-3-oxo-2H-1.4-benzoxasine-2-acetate	

Table 3.1. Chemical structure of newly synthesized compounds

3.2. Methods

3.2.1. Solubility of Compounds

Solubility of compounds in solvents is an important parameter for not only reverse transcription inhibition experiments but also to test cytotoxicity and cell proliferation effects on PBMC. Tested solvents were DMSO, ethanol, methanol and lysis buffer of reverse transcriptase assay kit. Each compound was investigated according to their behavior in these solvents and also their dilutions in lysis buffer. Because of the importance of lysis buffer for normal work of reverse transcriptase assay

kit, first solubility test was applied by using lysis buffer. Secondly, solubility of compounds in ethanol, methanol and DMSO were investigated. 1 mg compound was tried to be solubilzed in 1 ml of pure solvent and pellet formation after centrifugation was detected for determination of solubility. Solubilized compounds were hence diluted in lysis buffer and RPMI 1640. These diluted samples were also tested for precipitation formation in the same way.

The chosen interval for reverse transcription inhibitory effect of compounds was in 0.01–100 μM. Four points in this interval were provided and tested for each compound. 60 μl reaction mixture contained 100 μM, 10 μM, 1 μM, 0.01μM concentrations in separate wells. Maximum 1000 μM minimum 0.01μM concentrations of compounds were selected for blood cell experiments. Experimental calculations were arranged to fix solvent concentration at 2 % to prevent any interference of inhibitory effect of used solvent on reverse transcriptase. For PBMC cultivation, this concentration was fixed at 1% solvent/well. For this purpose, pure solvent serial dilutions were prepared, then equal amounts of each concentration were pipetted to add lysis buffer or RPMI 1640 according to experimental procedure. All compounds were solubilized separately before each experiment was done, then stored at –20°C.

3.2.2. Determination of Reverse Transcriptase Inhibition

Reverse transcriptase inhibition effects of compounds, DMSO and ethanol were measured by using non-radioactive ELISA kit from Roche. The detection and quantification of synthesized DNA as a paremeter for RT activity follows a sandwich ELISA protocol and the absorbance of incorporation of digoxigenin and biotine-labeled dUTP into DNA is directly correlated to the level of RT activity in the sample. Assay was performed as described in the protocol included with the kit, except for one hour reverse transcriptase incubation step. Briefly, the initial step of the assay is binding of biotin and dioxygenin labeled DNA on to the surface of pre-streptovidin coated microtiter plate. In the next step, an antibody to dioxygenin, conjugated to peroxidase (anti-DIG-POD), binds to the dioxigenin-labeled DNA. In the final step the peroxidase substrate ABTS is added. The peroxidase enzyme catalyzes the cleavage of the substrate, producing a colored reaction product. In the experimental section, 20 μl (4ng/well) recombinant reverse transcriptase enzyme diluted in lysis buffer was pipetted into a streptovidin coated microtiter plate per well. As a negative control, 20 μl lysis

buffer with no RT was used. Other control was included only RT, no inhibitor. Subsequently, 20 µl inhibitor diluted in LB, 20 µl reaction mixture including templete and nucleotides were added. Total volume was 60 µl. After 2 hours incubation at 37°C, plates were washed with 250 µl washing buffer and added 200 µl of anti-dioxigenin-peroxidase (Anti-DIG-POD, 200 mU/ml). Following 1 hour incubation at 37°C, plates were washed 5 times again and 200 µl ABTS substrate solution/well was added, incubated on orbital shaker at 75 rpm, room temperature until sufficient color development obtained. Optical density (OD) was measured by microtiter plate (ELISA) reader at 405 nm (reference wavelength: 490 nm). Results were expressed as % inhibition. This test is the first step for new compounds to find if the compound is effective on target or not. Activity of the compounds is expressed as IC_{50} (50% inhibitory concentration or concentration required to inhibit HIV-1 RT activity by 50%) valu (De Clercq, 1998). Percent inhibition of RT enzyme was calculated as the formula shown below.

% RT inhibition = 1 - [(OD of the Sample – Unspecific Binding)/(OD of the Control– Unspecific Binding)]

3.2.3. Isolation of Peripheral Blood Mononuclear Cells

Current law demands that new drugs, cosmetics, food additives, implants etc. should be tested by cytotoxicity tests before they are evaluated clinicaly. *In vitro* tests are not completely related with *in vivo* activity but can give selective mode of action for a compound. In addition, *in vitro* cytotoxicity tests are now used as an alternative method to animal experiments. In a drug discovery pipeline, cell viability (dye exclusion, cell counts), cell metabolism (dye reduction), cell replication (cell proliferation) can be investigated. Cytotoxicity assays should be set according to target response of cells to the chemicals and blood cells serve as a good source for body response, in other word, peripheral blood is the primary source of lymphoid cells for the exemination of immune response in humans. Isolated PBMCs contain 5 % B lymphocytes, 5-15 % monocytes, 60-70 % T lymphocytes and 5-15 % Natural Killer Cells (Reichert et al., 1991). Ficol hypec gradient centrifugation was used for isolation of PBMCs. While Ficoll sucrose polymer aggregates the eritrocytes in the bottom, low

dencty PBMC and platelets form a layer on the top of gradient. Platelets are removed by washing with PBS.

Heparinized blood was collected from healthy, non-smoking men and women of age between 24-30 as described in literatures (Hokland M. *et al*., 1994). Blood samples were diluted with equal volume of 0.9 % NaCl. 5 ml diluted blood was spreaded onto 3 ml ficol in a conical falcon tubes then cetrifuged at 1600 rpm, 25 °C for 25 min. Buffy coat was drived into new tubes by sterile pastor pipet and washed with 10 ml sterile phosphate buffered saline (PBS) solution containing 2 % FBS at 800 rpm for 10 min. After removing the supernatant by pouring, cells were washed 2 times with PBS at 500 rpm for 8 min to remove platelets and thrombocytes. The cells were collected into one tube with 2 ml RPMI containing 15 % FBS, 50 μg/ml gentamycin solution. 50 μl cell suspension was diluted with 50 μl trypan blue solution (0.4 %) then counted with 25 square hemocytometer under light microscope, and seeded into microtiter plates at 2 x 10^5 cell/well density. This formula was used to reach total cell number:

Number of cells/ml = Number of cells counted in 25 square x Dilution factor x 10^4

3.2.4. The Viability of PBMCs

Totxic effects of the compounds (AVM 001, AVM 002, AVM 010, AVM 011, AVM 014, AVM 015, AVM 019) on the viability of PBMCs were determined by trypan blue dye exclusion method. The method depends on a breakdown in membrane integrity. However a viable cell is impermeable for uptake of a dye, dead cells become permeable and can be distinguished from live cells by their blue color under light microscope. This methot is simple method for the determination of the anti-HIV-1 drugs CC_{50} value (CC_{50} is the inhibitory concentration that reduces cellular growth or viability of uninfected cells by 50%) (Lin T.S. *et al*., 1988). Cells were seeded with filter sterilized solution of compounds in 1000 μM-0.01 μM concentration range in 96 well flat bottom tissue culture treated plates. Six points of this range were 1000μM, 500 μM, 100 μM, 10 μM, 0.001 μM. Cells were counted at 24 h, 48 h, and 72 h drug exposure periods. At the end of time periods cells were collected slowly in eppendorfs and dead and live cells were counted under light microscope with hemocytometer just after mixing 50 μl cell suspension and 50 μl trypan blue. The following formula was used to calculate cell viability:

Percent Cell Viability = [Number of live cells / Number of total cells (live+dead)] x 100

3.2.5. The Proliferation of PBMC

Effect of compounds AVM 001, AVM 002, AVM 010, AVM 011, AVM 014, AVM 015, AVM 019 on proliferation of PBMCs was determined by using cell proliferation ELISA system, version 2 from Amersham Life Science. This assay depends on the incorporation of pyrimidine analogue BrdU into DNA of proliferating cells instead of thymidine. Peroxidase labelled anti-BrdU binds to BrdU in newly synthesized cellular DNA. Color formation is achieved after addition of substrate and absorbance is measured at 450 nm by ELISA plate reader. Isolated PBMCs were incubated for 48 h in a CO_2 incubator at 37 °C in a humidified atmospher with compounds. At the end of incubation period, BrdU was added and reincubated. After 16 hours, 96 well plate was centrifuged at 1200 rpm for 15 min, labelling medium was removed by gently tapping and cells were dried by hair drier for 15 min. 200 µl fixative/well added plate was incubated 60 min at room temperature. After 200 µl blocking reagent/well was added 30 min incubation period followed by removing solution by tapping and pipetting 100 µl peroxidase-labelled anti-BrdU. 2 h incubation period at room temperature was applied before removing unbound substances and 3 times washing with washing buffer. 100 µl room temperature equilibrated TMB substrate solution/well was added and plate was incubated in an orbital shaker at RT until required color density was achieved. Color reaction was stopped by pipetting 25 µl of 1 M sulphuric acid. Optical density of the samples was measured by ELISA plate reader at 450 nm in 5 min.

3.2.6. AMES Mutagenicity Test

Salmonella mutagenicity test (AMES) developed by Bruce N. AMES was used to determine mutagenic potential of compounds (AVM 001, AVM 002, AVM 010, AVM 011, AVM 014, AVM 015, AVM 019). AMES test depends on the histidine requirements of the *Salmonella typhimurium* strains becacause of the mutation in histidine operon. This is because tester strains require histidine to grow. If the strain undergoes the beck mutation in the His gene because of the carcinocens it does not require histidine anymore. Two histidine-requering strains, TA 100 and TA102 that

contain the R-factor plasmid were used to determine mutagenic potential of compounds. In addition to R-factor plasmid that increases permeability of cell membrane for large molecules because of the partial loss of the lipopolysaccharide barrier, TA102 contains PAQ1 mutation which carries the hisG428 mutation and a tetracycline resistance gene. PAQ1 mutation can be utilized to detect oxidative mutagens in contrast to the other Salmonella tester strains that detect organic carcinogens. Since the plasmid is somewhat unstable and can be lost from the bacteria, strains were tested in the presence of the ampicillin. The PAQ1 strain (TA102) was tested for both ampicillin and tetracycline resistance on ampicillin/tetracycline plates. Reverse mutations of tester strains that show resistancy to antibiotics were detected for their histidine dependence or independence.

Tester strains TA 100 and TA 102 were grown in nutrient broth no: 2 in 50 ml flasks at 37 °C with shaking overnight in thermal shaker for 16 hours as a first step. In order to prevent back-mutation cultures were protected from sunlight by covering aluminium foil during incubation and experiment. Compounds were solubilized in DMSO and tested at 0.01µM, 0.1µM, 1µM, 100µM, 1000µM and 3000µM concentrations per plate. Next day, 0.1 ml growing culture, 0.5 ml PBS (pH: 7.4) and 0.1 ml compound were pipetted in sterile test tubes and mixed slowly by vortex than pre-incubated for 1 hour at 37°C with shaking as asecond step. After preincubation period, 2 ml top agar containing 0.5 mM Histidine-Biotin was added into tubes, vortexed slowly for 3 seconds and poured onto glucose agar plates. Poured mixture was uniformly distributed on plate surface. Preperation of pour plate were completed in 20 seconds. Duplicate plates were incubated at 37°C for 48 hours and the revertant colonies with background lawn were counted.

Plates containing no compound as a negative control, 5 ml sodium azide (17.2 mg/ml) as a positive control, and only biotin were compared to determine the mutagenic potentials of compounds.

3.2.7. The Activation of PBMC (MTT Assay)

The *in vitro* tetrazolium-based colorimetric assay (MTT) was used to detect the activation of PBMC. This assay also represents cytotoxic effects of the compounds AVM 001, AVM 002, AVM 010, AVM 011, AVM 014, AVM 015, AVM 019. Method is based on the cleavage of a yellow tetrazolium salt 3-{4,5-dimethylthiazol-2-

yl}-2,5 diphenyl tetrazolium bromide (MTT) to purple formazan crystals by mitochondrial enzymes of metabolically active cells.

Cells were seeded with filter-sterilized compounds described in cell viability for 24 hours, 48 hours, and 72 hours at 37°C with 5% CO_2 humidified incubator. Three hours before the end of each incubation period, MTT stock solution (5 mg/ml) was added into each well at 1:10 ratio and incubated then plates were centrifuged at 1800 rpm for 10 minutes at room temperatures in a plate rotor of Hettich 30 RF Centrifuge to avoid accidental removal of formazan crystals. Supernatant removing by tapping on a paper towel was followed DMSO (150 µl) addition and mixing on an orbital shaker at 150 rpm for 5 minutes. The absorbance values were determined at 540 nm (690 nm reference wavelength) by using an automated plate reader (Organon Teknica Reader 230S, Version 1,22).

Chapter 4

RESULTS AND DISCUSSION

4.1. Solubility Properties of the Compounds

Nineteen newly synthesized compounds (structures of compounds were given in Materials and Methods section) were examined in Lysis Buffer (LB) of reverse transcriptase RT assay, DMSO, ethanol or in methanol and also their 100 % solubilized form were diluted in Lysis buffer (LB) and RPMI 1640 to detect precipitate formation.

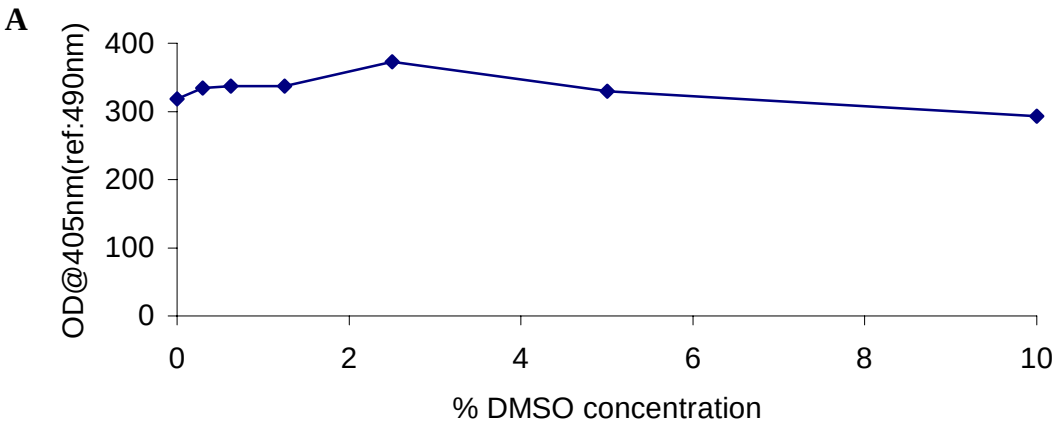
First 1 mg compound was solubilized in 500 µl of LB. Except AVM 017; none of the compounds were solubilized in LB. Because of the high inhibitory effect of the Ethanol on RT, it was not preferred as a first choice for solubilization of compounds. Therefore, it was only chosen for compounds, which was not dissolved in DMSO. In all experiments AVM 001 and AVM 002 were dissolved in ethanol. Remaining compounds were solubilized in DMSO. AVM 008 and AVM 016 became solubilized after long time vortexing. All dissolved compounds in 100 % solvent formed precipitation when over 500 µM concentration were used to dilute in RPMI. Solvent concentration was fixed at 2 % for all RT inhibition experiments, 1 % for all cell culture. Solubilization properties of compounds in 100% solvent were given in Table 4.1.

Compound	Lysis buffer	Methanol 100%	Ethanol 100%	DMSO 100%	Compound	Lysis buffer	DMSO 100%
AVM 001	-	+	+	-	**AVM 011**	-	-
AVM 002	-	+	+	-	**AVM 012**	-	-
AVM 003	-	+	+	+	**AVM 013**	-	-
AVM 004	-			+	**AVM 014**	-	-
AVM 005	-			+	**AVM 015**	-	-
AVM 006	-			+	**AVM 016**	-	-
AVM 007	-			+	**AVM 017**	+	+
AVM 008	-			+	**AVM 018**	-	-
AVM 009	-			+	**AVM 019**	-	-
AVM 010	-			+			

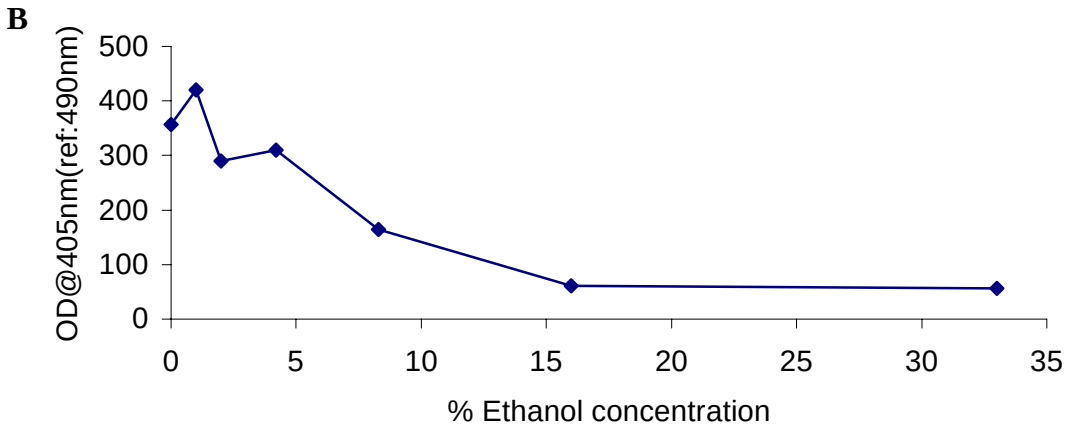
Table 4.1. Solubilization properties of compounds in different solvents.

4.2. Inhibitory Effects of Compounds on Reverse Transcriptase Enzyme Activity

Before the examination of compounds on RT, RT inhibition effects of ethanol and DMSO were determined. Figure 4.1.A shows that DMSO in range of 0-10 % did not have any effect on the activity of RT enzyme. However, the inhibitory effect of ethanol on RT enzyme given in Figure 5.1.b started at 2 % concentration and inhibitory effect gradually increased until 16% of ethanol and stayed at the same level in the presence of 33 % ethanol concentration (Figure 4.1.B). It was found that ethanol had higher inhibition effect than DMSO. Thus, DMSO was preferred as a first choice to solubilize the compounds (Barnard et al., 1997, Xie *et al*., 1999, Hashimoto *et al*.,1997).



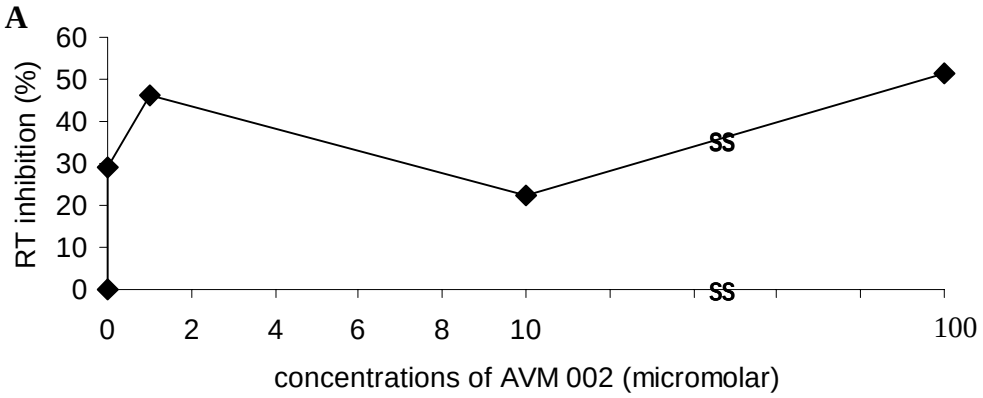
RT inhibitory effect of DMSO



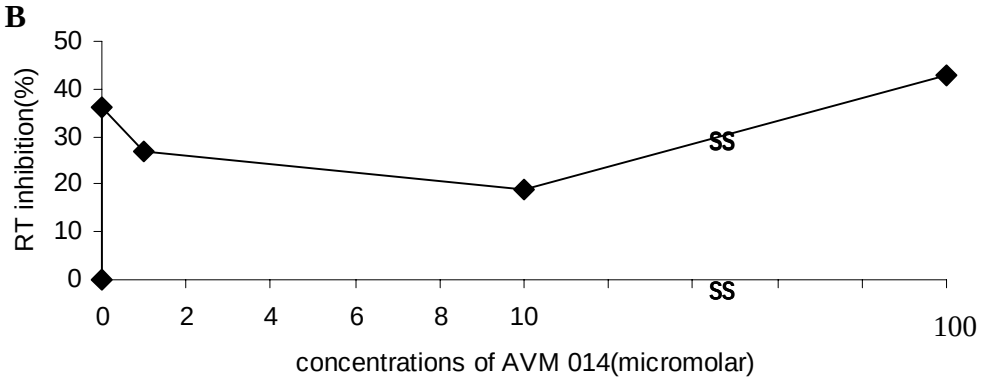
RT inhibitory effect of ethanol

Figure 4.1. RT inhibition effect of DMSO (A) and ethanol (B). The experiment was carried as duplicate. The difference between OD values of each duplicate sample was less than 5%.

Compounds, dominantly designed as benzaxozale derivatives especially AVM 002 [5-methyl-2-(fenoksymethil)benzaxozole] and AVM 014 [2-(p-florophenil)-5-aminobenzaxozole] at 100 µM concentration showed 51.5 % and 43 % inhibitory effect respectively, Figure 4.2.A shows inhibition effect of AVM 002 on RT enzyme. AVM 002 concentration at 0.01µM resulted in 29 % enzyme inhibition and inhibition at 1 µM was very close to 50 % inhibition. In the dose-response graph a decline to 22.5% in the inhibition was observed at 10 µM concentration. However, % RT inhibition was reached to 51.5% at 100 µM concentration point of AVM 002. As it can be seen in Figure 4.2.B, AVM 014 also showed good inhibition effect on RT enzyme. Percent inhibition of RT enzyme activity increased to 26.9% at 1µM concentration. However, percent inhibition decreased at 10µM to 19% inhibition and, and reach 43 % inhibition at 100µM concentration.



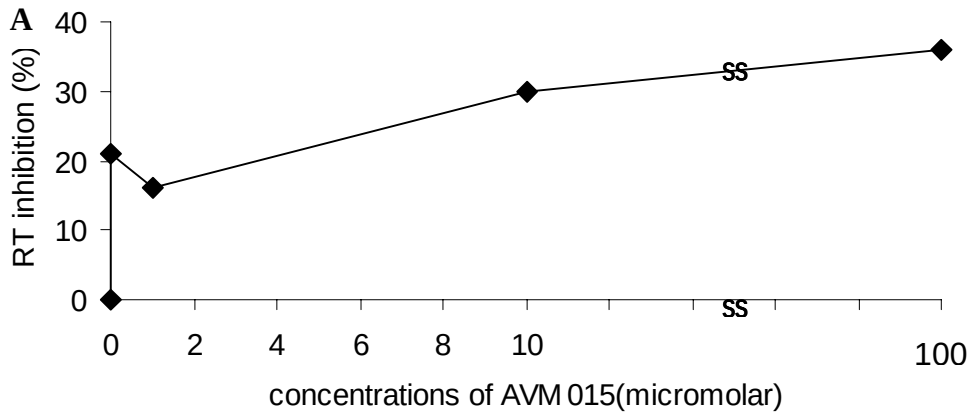
Precent inhibitory effect of AVM 002 on RT



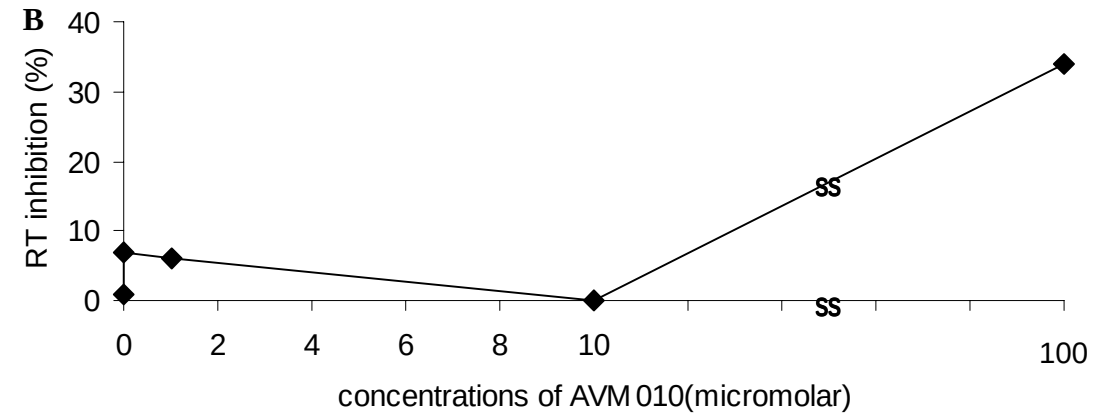
Percent inhibitory effect of AVM 014

Figure 4.2. Inhibitory effect of AVM 002 (A) and AVM 014 (B) on RT. Data are the average of separate three experiments for AVM 002 and separate two experiments for AVM 014. (Standard error of each point is given in table 4.2).

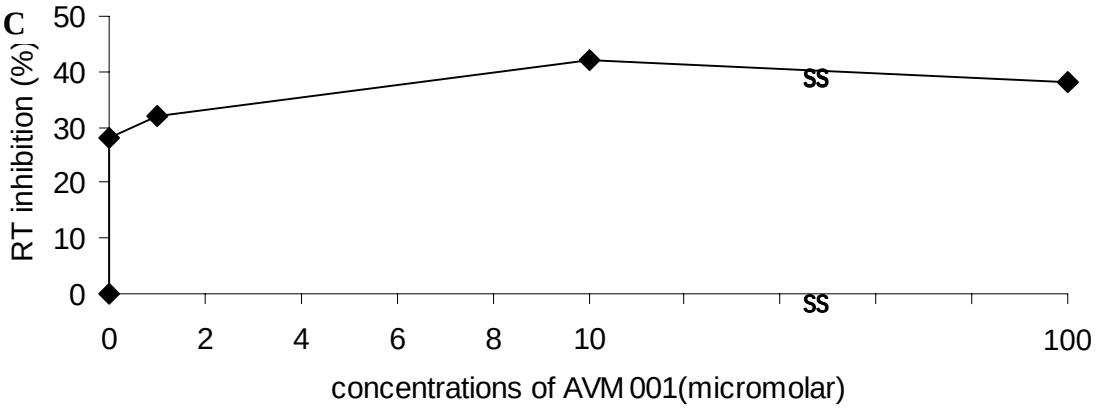
Figure 4.3. represents dose-response curves of AVM 015, AVM 010, AVM 001, AVM 011 and AVM019. When concentration of AVM 015 was increased, inhibition rate increased and reached 30% and 36% inhibition at 10μM and 100μM of AVM 015, respectively (Figure 4.3.A). Figure 4.3.B shows inhibition effect of AVM 010 on RT enzyme. Inhibition effect of the compound was found around 5 % at 0.01 μM, 1 μM and 10 μM concentrations but inhibition effect of AVM 010 on RT enzyme picked to 34.3 % inhibition at 100 μM compound concentration. Dose-response curve of AVM 001 was given in Figure 4.3.C. At 0.01μM compound concentration; percent inhibition was at 28 %. It was sharply increased with 1μM and 10μM concentrations and reached to 32% and 42% inhibition. At 100 μM, there was decrease in inhibition effect on RT enzyme to 38 % was obtained. Inhibition effect of AVM 011 showed gradually increase at compound concentration from 0.01 μM and continued through to 1 μM, 10 μM and reach to 7.4%, 17.9 % and 25.8 % respectively. This increase stopped at 10 μM concentration reached 25.3 % RT inhibition effect (Figure 4.3.D.) at 100μM. According to results obtained from experiments with AVM 019, the inhibitory effect on the enzyme picked to 23.8 % at 1 μM compound concentration. The inhibitory effect of the compound at 10 μM decreased to 17% and followed by an increase at 100 μM compound concentration to 27.6 % RT inhibition (Figure 4.3.E).



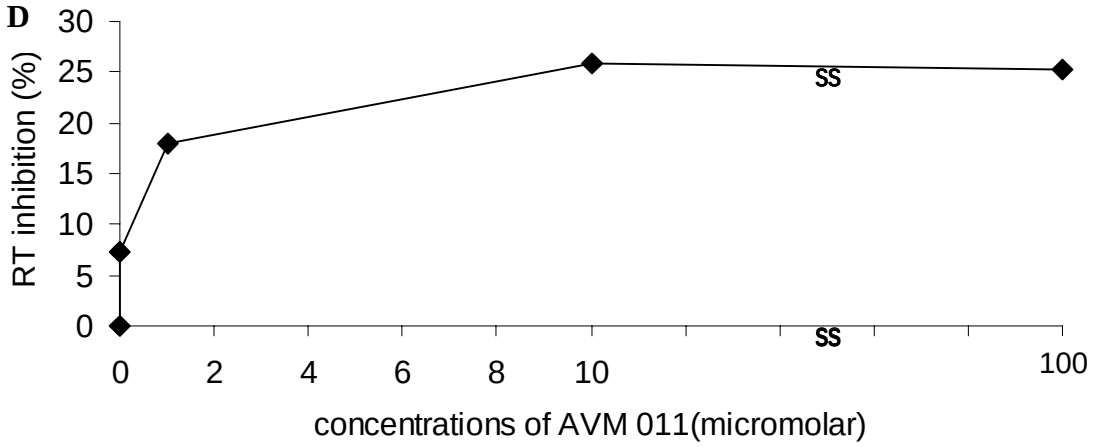
Percent inhibitory effect of AVM 015



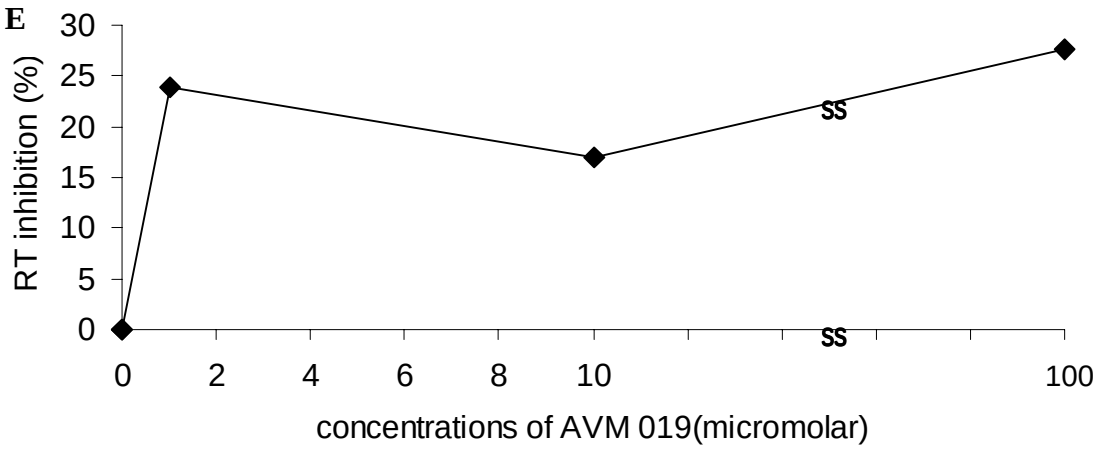
Percent inhibitory effect of AVM 010



Percent inhibitory effect of AVM 001



Percent inhibitory effect of AVM 011



Percent inhibitory effect of AVM 019

Figure 4.3. Percent inhibitory effects of AVM 001 (A), AVM 010 (B), AVM 011 (C), AVM 015 (D) and AVM 019 (E) on RT activity. Data are the average of separate three experiments for AVM 001 and AVM 011, separate two experiments for AVM 010, AVM 015 and AVM 019. (Standard error of each point is given in table 4.2).

Meaningfull RT inhibition effects were not observed for another compounds. Therefore, they were not used rest of the experiments in this study.

Table 4.2 shows summary of the effects of the compounds on RT activity and percent variation between separate experiments. High variation between separate experiments depends on varying assay conditions for example unstable laboratory temperature, as it reported in the literature (De Clercq, 1998). Determination of the EC_{50} (50% effective concentration, or required to inhibit HIV-1 induced cytopathicity) values can show utility of the compounds for HIV-1 (+) patient.

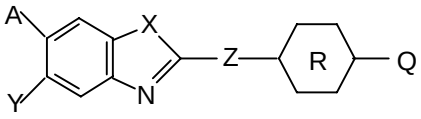
	Concentration				
Compounds	**0**	**0.01**	**1**	**10**	**100**
AVM 001	0	28.2±4	32.2±8	42±8	38.4±6
AVM 002	0	29±5	46±7	22.5±10	51.5±3
AVM 010	0	7±1	6±1	0±0	34.3±6
AVM 011	0	7.4±0	17.9±2	25.8±5	25.3±8
AVM 014	0	3.6±2	26.9±2	19±6	43±5
AVM 015	0	21±3	16±5	30±2	36±3
AVM 019	0	0±0	23.8±5	17±6	27.6±4

Table 4.2. Summary of the % inhibition effects of the compounds on RT activity and percent variation between separate experiments (Results are percent inhibitory effect).

FDA approved NNRTIs Nevirapine, Dalavirdine, and Efavirenz (Benzoxazinone) have 0.084 μM 0.26, μM and 0.003 μM IC_{50} (50 % inhibitory concentration, or concentration required to inhibit HIV-1 RT activity by 50 %) respectively (De Clercq, 1996, Merluzzi *et al*., 1990, Koup, *et al*., 1991, Dueweke, *et al*., 1993, Young, *et al*., 1995). It is important to note that for all of these compounds whether approved or compounds in clinical trials generally show highly reduced EC_{50} (50 % effective concentration, or concentration required to inhibit HIV-1 induced cytopathicity by 50 %) value when compared with IC_{50}. This manner means that activity of newly synthesized compounds can be observed at reduced concentration when assayed with infected cell lines. In addition, compounds that did not show inhibitory activity on HIV-1 RT can be active for resistant strains.

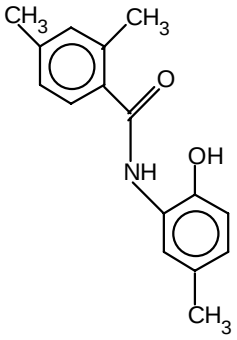
Chemical structure of benzaxazole is given in Figure 4.4. As it can be seen AVM 002 seems to fit better for hydrophobic pocket of HIV-1 RT than the others when they were compared with the other compounds showed inhibitory effect on RT, especially AVM 010. CH_3 group at Y position gave the best result. In addition, AVM 0014 also showed IC_{50} value at 100μM concentrations and for this compound N_2H molecule at Y position and F atom at Q position resulted with high inhibition effect on RT enzyme.

Oxygen at X position found in benzaxozoles seems to be necessary to get high HIV-1 RT inhibition effect when the inhibitory activities of benzaxazoles is compared with benzymidazoles, because AVM 010 that belongs to benzaxozole group and AVM 013 from benzimidazole group have the same structure except substitution of X. AVM 010 has oxygen atom in this position instead of NH and showed high inhibition. AVM 007 also is benzimidasole and has close structure with AVM 010 but Q group is an hydrogen atom. We suggest that Cl group in Q position is not important for RT inhibition. AVM 015 and AVM 019 have different structure than other tested compounds (Figure 4.5).



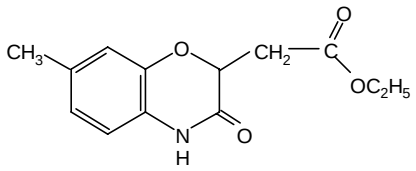
	Y	A	X	Z	Q	R
AVM 001	Cl	H	O	-	CH_3	O
AVM 002	CH_3	H	O	$-CH_2-O-$	H	O
AVM 010	H	H	O	$-CH_2-O-$	Cl	O
AVM 011	H	O_2N	O	-	H	-
AVM 014	H_2N	H	O	-	F	O

Figure 4.4. Substitutions in benzaxazole derivatives.



N-[(2-hidroxy-5-methyl)phenyl]-
2,4-dimethyl-bensamide

AVM 015



Ethyl 3,4-dihydro-7-methyl-3-oxo-2H-
1.4-benzoxasine-2-acetate

AVM 019

Figure 4.5. Structures of AVM 015 and AVM 019.

Since there are no structurally related compounds with newly synthesized benzaxazoles and benzimidazoles and determined RT inhibitory activities in literature, comprehensions were done by using data obtained from this study and literature of FAD approved compounds.

4.3. Effect of Compounds on Cell Viability of PBMC

Compounds that showed RT inhibition between 20-50 percent were examined to determine the effects on viability of PBMC. Concentration points at 1000 µM, 500 µM, 100 µM, 1 µM, 0.1 µM and 0.01 µM per well were used to plot the dose response curve. Dilutions were set and calculated to obtain 1% solvent concentration per well. All dilutions were prepared just before the experiments. Stored dilutions were not used to avoid any change in compound structure. 2 x 10^5 cells per well were used. And cells were incubated in the culture medium containing compound at different concentrations for one, two or three days in order to see if compound has any toxic effect on cell viability. Culture medium with the cells without compound served as control in all viability experiments.

AVM 001 caused little decrease in cell viability at the end of first 24 hours at all concentrations examined. There was only 12% decrease in cell viability of cells incubated with 0.01µM concentrations of AVM 001. For 48 and 72 hours, 25 % and 29 % decline were observed in the wells having 1000 µM compound concentration (Figure 4.6).

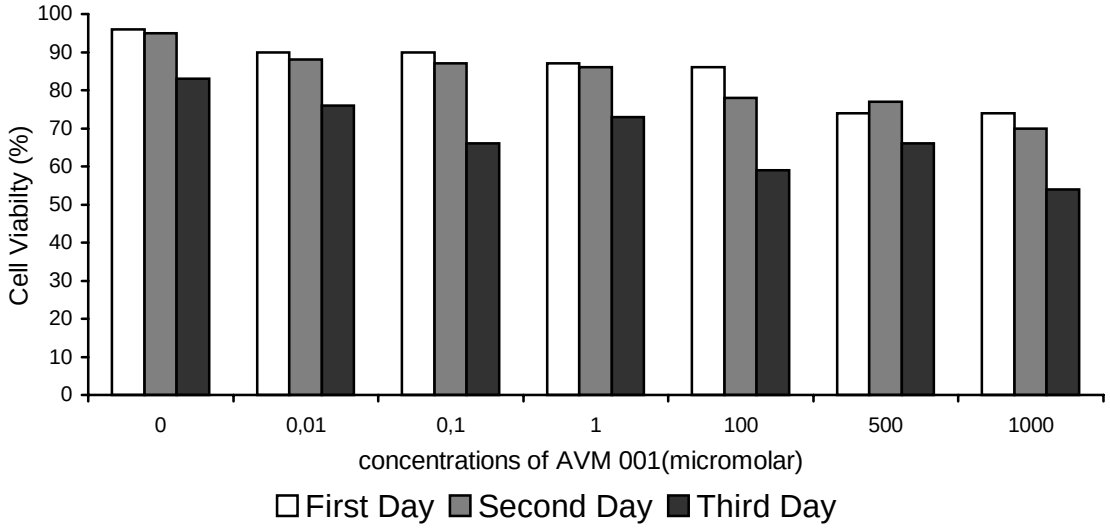


Figure 4.6. Effects of AVM 001 on the cell viability of PBMC. Data are the average of two different experiments.

The compound AVM 002 did not affect cell viability at concentrations from 0.01µM to 100µM (Figure 4.7). There was a sharp decrease in the cell viability at 500µM and 1000µM concentrations. 36%, 50%, and 48% decrease in cell viability was

observed with 500µM compound at the end of day 1,2, and 3, respectively. Similarly, 1000µM compound resulted in 48%, 54%, and 54% decrease in cell viability at the end of day 1,2, and 3. These results imply that compound AVM 002 has toxic effect on the cell viability at 500µM and 1000µM concentrations, compared to control.

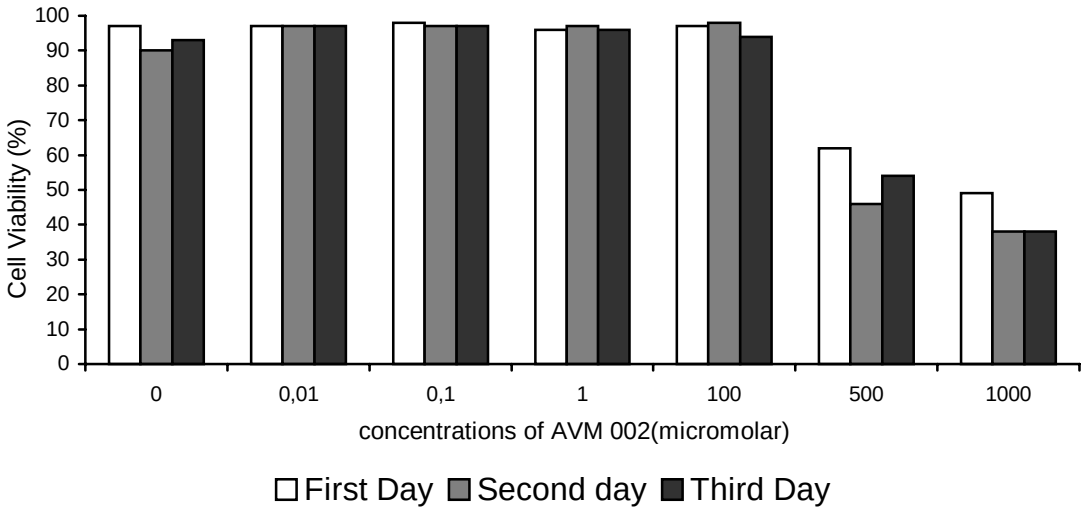


Figure 4.7. Effects of AVM 002 on cell viability of PBMC. Data are the average of two different experiments.

As with similar to compound AVM 002, the compound AVM 014 did not show toxic effects on the cell viability at concentrations from 0.01 to 100µM, compared with controls (Figure 4.8). When the results obtained from day 1 at all concentrations are compared to each other, viability stayed almost at the same level, around 90%. Similar pattern of effect on cell viability was observed at the end of day 2 and 3 until compound concentration increased to 100µM. However, cell viability decline to about 40% at the end of day 2 and 3 in the presence of compound at 500µM and 1000µM concentration. Taken together, AVM 014 had toxic effect at 500µM and 1000µM concentrations.

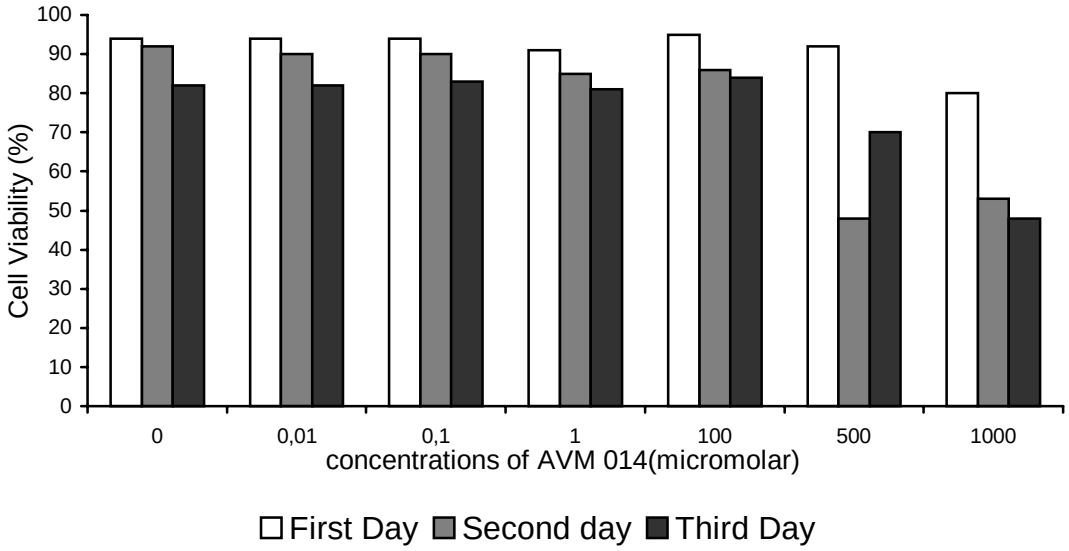


Figure 4.8. Effects of AVM 014 on the viability of PBMC. Data are the average of two different experiments.

Even though AVM 010 showed similar pattern with other compounds until 100µm concentration, it was the most toxic one when compared with the others at 500µM and 1000µM concentration where cell viability was observed to be less than 40% at the end of day 1, 2, and 3 (Figure 4.9).

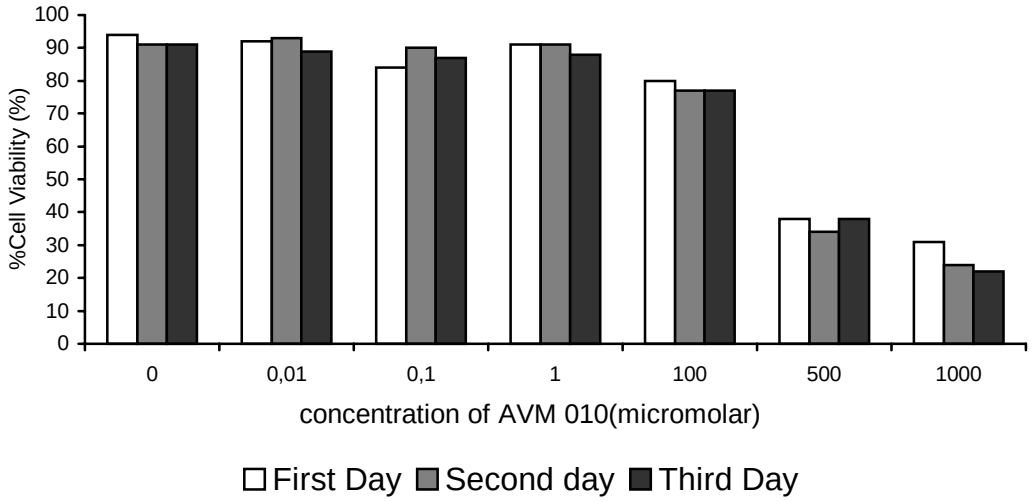


Figure 4.9. Effects of AVM 010 on the cell viability of PBMC. Data are the average of two different experiments.

The cell viability was around 80% and more at all concentrations of AVM 011 compound at the end of day 1, 2, and 3 (Figure 4.10). The decrease in the viability was around 19% even at 1000µM concentration, compared to the control. Therefore, AVM 011 was less or not toxic at 500µM and 1000µM concentrations when it was compared with previous compounds.

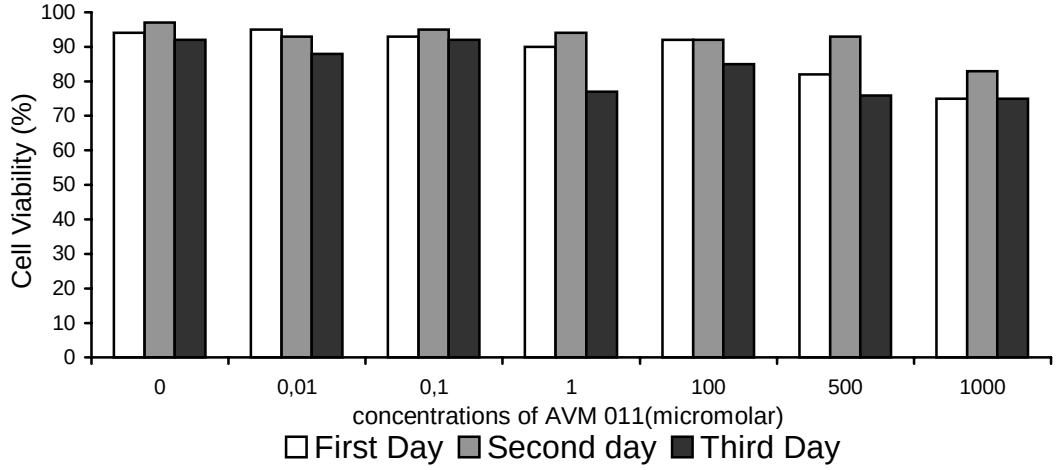


Figure 4.10. Effect of AVM 011 on cell viability of PBMC. Data are the average of two different experiments.

The compound AVM 015 did not show any effect on the viability of PBMC. For all three days,cell viability was found over 90 % (Figure 4.11), indicating that AVM 015 is not toxic on cell viability.

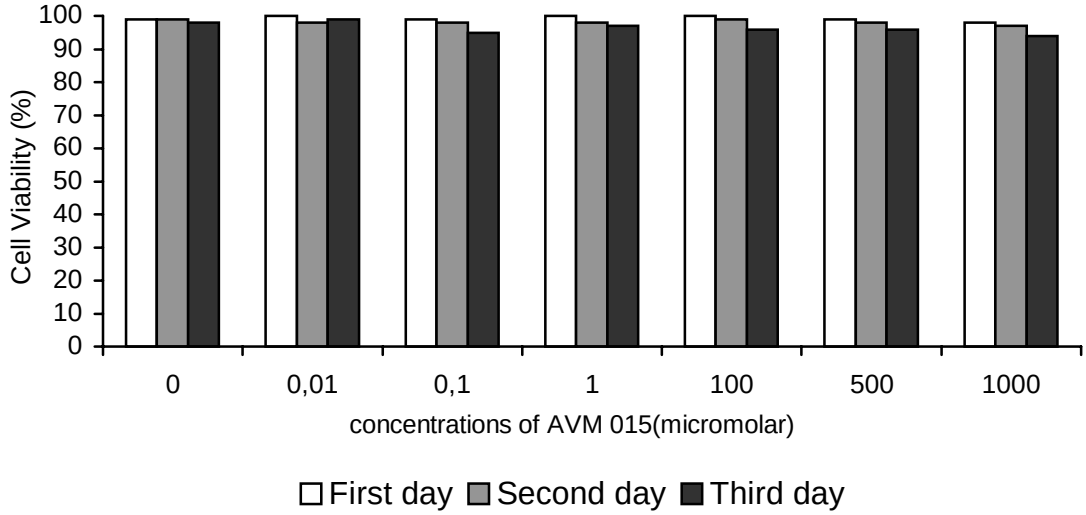


Figure 4.11. Effect of AVM 015 on cell viability of PBMC. Data are the average of two different experiments.

The viability of PBMC treated with AVM 019 did not change after treatment with the compound until 1000μM concentration at the end of day 1, 2 (Figure 4.12). However, there was 28% and 48% decrease in the cell viability after day 2 and 3 at 1000μM compound concentration.

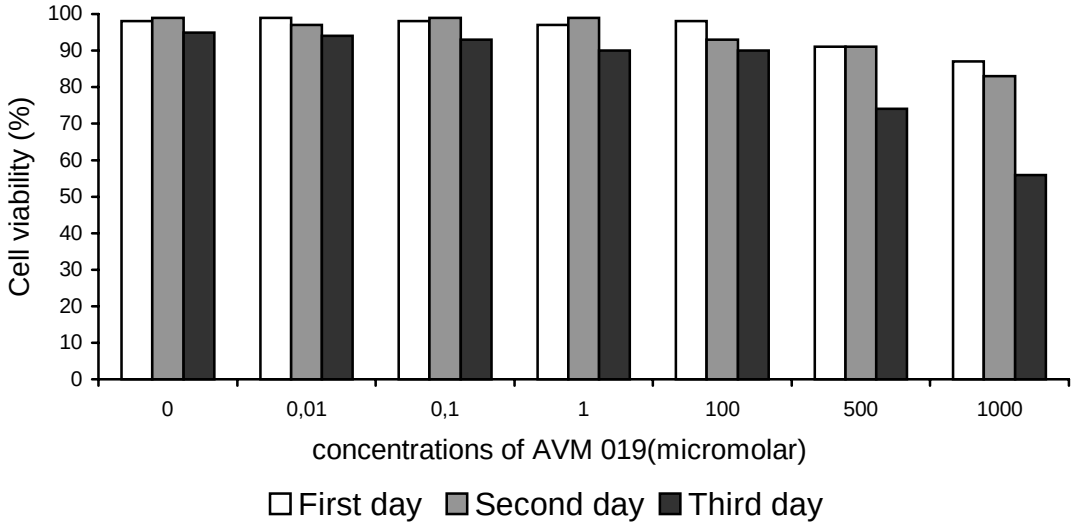


Figure 4.12. Effect of AVM 019 on cell viability of PBMC. Data are the average of two different experiments.

It is expected that the therapeutics should reach to desired beneficial effect with minimal adverse effects. Clearance (the measure of the ability of the body to eliminate the drug) is the basic parameter for the drugs. Elimination of drug from the body may involve process occurring in kidney, lung, liver and other organs. While no chemicals can be certified as completely "safe" (free of risk) since every chemical is toxic at some level of dosage, it is possible to estimate the risk associated with exposure to the chemical under specified conditions if appropriate tests are performed. The toxic effect of the compounds may be due to the compound structure, accumulation of compound in cells especially in high dosage contained wells, or accumulation of the metabolite especially at the end of 48 and 72 hours. In addition, half-life and the clearance of the compounds are unknown. Moreover, *in vitro* experiments are close systems and also metabolite removal or refreshment of the medium is not applied in short-term cytotoxicity tests. So decreased *in vivo* toxicity profile can be expected for the tested compounds. FDA approved NNRTIs Nevirapine, Dalavirdine, and Efavirenz (Benzoxazinone) have >50 μM, >100 μM and 80 μM CC_{50} (50% cytotoxic

concentration, or concentration required to reduce viability of the host cells by 50 %) respectively (De Clercq, 1996, Merluzzi *et al*., 1990, Koup, *et al*., 1991, Dueweke, *et al*., 1993, Young, *et al*., 1995). CC_{50} values of the tested compounds were higher than or equal to 500μM concentration. When the CC_{50} values of the FDA approved compounds are compared with the CC_{50} values of AVM 001 (>1000μM), AVM 002 (CC_{50}=500μM), AVM 010 (between 100μM-500μM), AVM 011(>1000μM), AVM 014 (CC_{50}=1000μM), AVM 015 (>1000μM) and AVM 019 (>1000μM), it can be easily seen that newly synthesized compounds have good cytotoxic profiles than Nevirapine, Dalavirdine, and Efavirenz. Table 4.3 shows the summary of the effects of the compounds on cell viability.

		Concentrations (μM)						
Compounds	**Days**	**0**	**0.01**	**0.1**	**1**	**100**	**500**	**1000**
AVM 001	**1**	96±2	90±0	90±0	87±4	86±1	74±5	74±4
	2	95±1	88±1	87±1	86±4	78±5	77±5	70±5
	3	83±1	76±1	66±1	73±4	59±5	66±5	54±5
AVM 002	**1**	97±2	97±1	98±0	96±2	97±1	62±2	49±2
	2	90±3	97±1	97±1	97±1	98±2	46±1	38±4
	3	93±1	97±0	97±0	96±4	94±2	54±1	38±5
AVM 010	**1**	94±4	92±1	84±5	91±1	80±4	38±4	31±5
	2	91±1	93±2	90±0	91±1	77±2	34±2	24±3
	3	91±3	89±0	87±1	88±3	77±3	38±3	22±3
AVM 011	**1**	94±3	95±1	93±2	90±2	92±1	82±5	75±1
	2	97±1	93±1	95±1	94±0	92±2	93±5	83±4
	3	92±1	88±3	92±	77±5	85±2	76±4	75±1
AVM 014	**1**	94±2	94±1	94±1	91±2	95±1	92±1	80±3
	2	92±1	90±4	90±3	85±2	86±4	48±5	53±5
	3	82±5	82±1	83±5	81±2	84±5	70±2	48±0
AVM 015	**1**	99±1	100±0	99±1	100±0	100±0	99±0	98±2
	2	99±1	98±2	98±0	98±1	99±1	98±1	97±3
	3	98±0	99±5	95±1	97±1	96±3	96±4	94±5
AVM 019	**1**	98±2	99±1	98±1	97±1	98±0	91±1	87±5
	2	99±1	97±5	99±1	99±2	93±2	91±5	83±1
	3	95±2	94±0	93±2	90±3	90±1	74±1	56±3

Table 4.3. Summary of the effects of the compounds on cell viability. Data are the average of two different experiments. Variation between two experiments was because of biological differences between two blood donors.

4.4. The Effects of Compounds on Proliferation of PBMC

Effects of compounds on the proliferation of PBMC were determined by using cell proliferation ELISA kit. Concavalin A (Con A) and phytohemoglutine (PHA) were used as a mitogen in the assay. 200 µg/ml of Con A and PHA that previously determined were used to obtain maximum proliferation of PBMC.

Effect of the compounds that showed inhibitory effect on RT, except AVM 015 and AVM 019 on the cell proliferation were determined by the cultivation of PBMC in the absence and presence of Con A or PHA. Increased optical density indicates that compound stimulates PBMC to proliferate.

AVM 001 caused 2.2 fold increase in cell proliferation when compared with the control at concentration of 1 µM. AVM 002 caused 2.5 fold increase at the same concentration. IC_{50} of AVM 002 was found at 100 µM and this concentration did not cause any change in cell proliferation when compared with control. However, 1000 µM concentration it had toxic effect on the cell viability. Same toxic effect was obtained at the same concentration for AVM 001 (Figure 4.13.A and B).

When PBMC were cultured with AVM 014, linear decline in the cell proliferation was obtained through the 1000 µM compound concentration (Figure 4.13.C). There was 82% decrease at 1000µM concentration compared to the control without AVM 014.

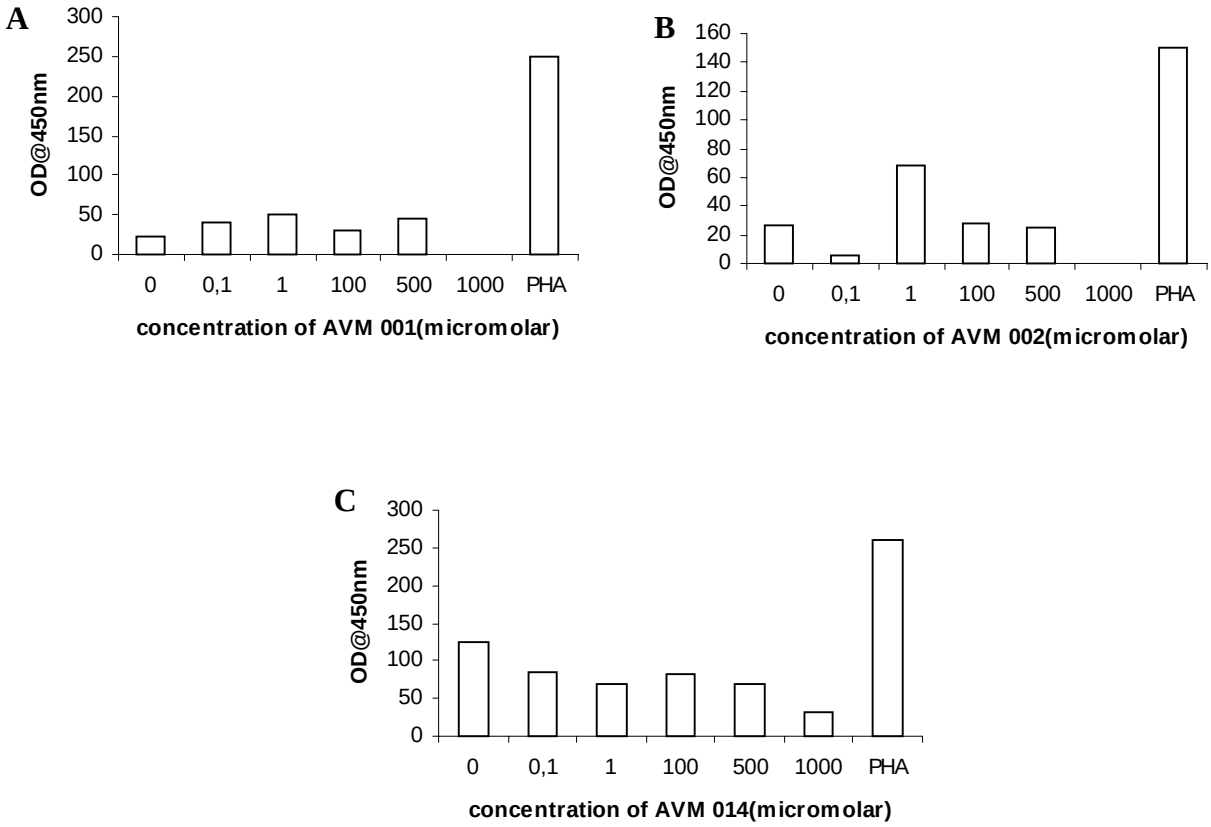


Figure 4.13. Effects of AVM 001 (A), AVM 002 (B) and AVM 014 (C) on proliferation of PBMC. Data are the average of duplicate samples.

AVM 010 (Figure 4.14.A) and AVM 011 (Figure 4.14.B) caused a decrease in cell proliferation when compared with control at all concentrations examined. At 100 µM concentration 2 fold decrease for AVM 010, 6 fold decrease for AVM 011 in cell proliferation were obtained. 100µM was the most toxic concentration of AVM 011 on cell proliferation when compared with the controls. In other words, AVM 010 and AVM 011 both had toxic effect on the cell proliferation. AVM 015 (Figure 4.14.C) gave totally different cell proliferation effect. Small concentrations of the compound caused decline in proliferation of PBMC but cell proliferation rate gradually increased at 500µM and 1000µM concentration. AVM 019 (Figure 4.14.D) appears to stimulate cell proliferation of PBMC compared to control (Table 4.4).

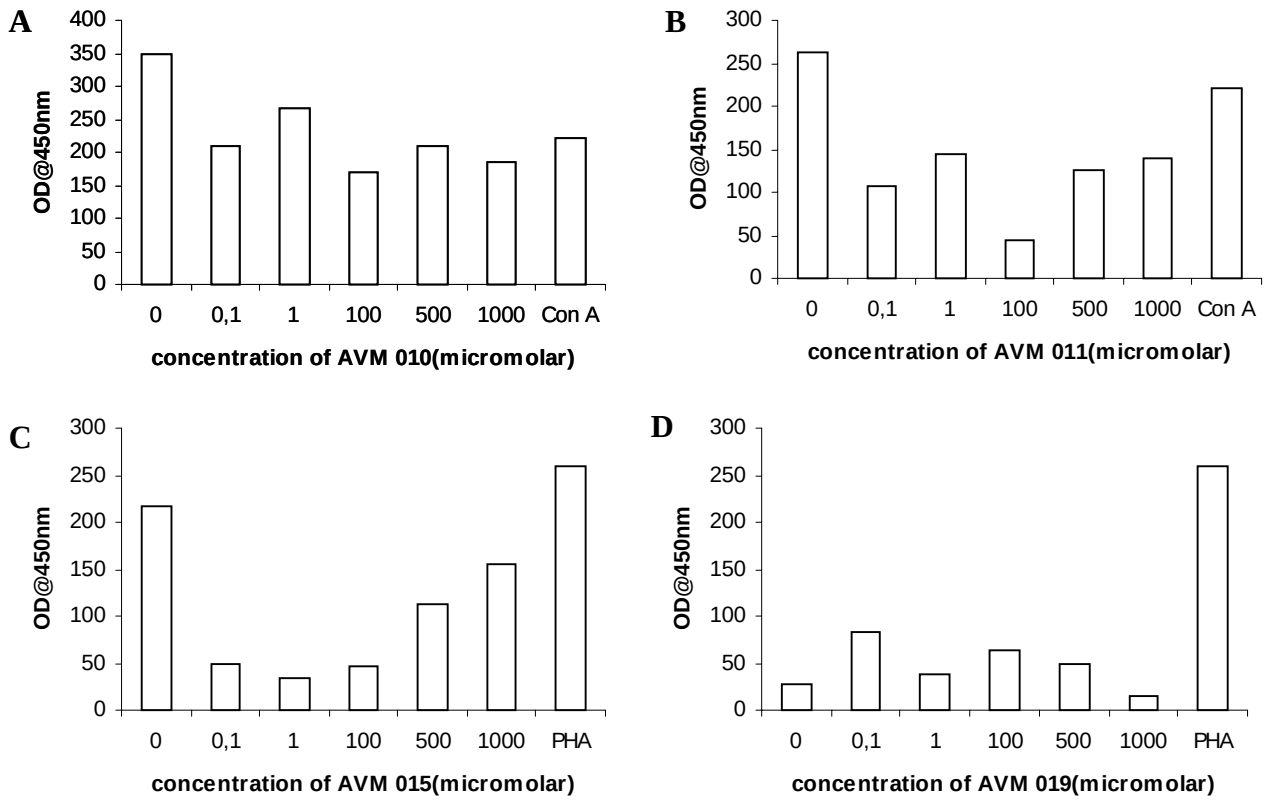


Figure 4.14. AVM 010 (A), AVM 011 (B), AVM 015 (C) and AVM 019 (D) on proliferation of PBMC. Data are the average of duplicate samples.

	Concentrations						
Compounds	**0**	**0.1**	**1**	**100**	**500**	**1000**	**Con A/PHA**
AVM 001	22.5	39.5	50	31	46.5	0	250
AVM 002	26.5	5	68	28	25	0	150
AVM 010	349	208	265	168.5	210	184	222
AVM 011	262	106	144	43.5	126	140	222
AVM 014	126	85	68	81	69	32	260
AVM 015	216	49	33	47	113	155	260
AVM 019	28	83	38	64	48	15	260

Table 4.4. Summary of the effects of the compounds on cell proliferation and percent variation between separate experiments. Results are average of OD values of duplicate samples. Differences between absorbance values of duplicate samples were less than 5% in all experiments.

HIV causes AIDS disease when immune system of a body is so damaged and immunological abnormalities start to occur. This manner means having less than 200 CD4+ cells. Additional decrease in cell proliferation due to use of drugs can cause increase in the probability of death because of the opportunistic infections especially at the last period of HIV infection. Therefore, AVM 010 and AVM 011 are not useful as an anti-HIV chemotherapeutics because they caused a decrease in cell proliferation. Change in the structure of AVM 014 should be focused to decrease the adverse effects on the biological parameters. Since AVM 019 caused an increase in cell proliferation, it seems to be safe according to this parameter. Other compounds require additional tests with infected cell lines. Because AVM 015 caused increase in cell proliferation at high concentration and has over 1000μM CC_{50} value, it can be useful in mega-HAART (Highly Active Antiretroviral Therapy) program. The aim of this therapy is to normalize the white blood cell count, decrease production of viral particles and make the immune system start to work by inhibiting the enzymatic pathways of HIV-1.

Other compounds examined on cell proliferation are AVM 003, AVM 004, AVM 005, AVM 006, AVM 007, AVM 009, AVM 012, AVM 013 and AVM 017. AVM 003 caused an increase (1.3 fold) at 1 μM concentration and 0.6 fold decrease at 100 μM concentration followed by small increase at 1000μM that was close to control. AVM 004 showed an increase in cell proliferation for all concentrations. In other words, AVM 004 had stimulatory effect on cell proliferation and can be developed as an immunostimulant. AVM 006 and AVM 007 did not show any significant change in cell proliferation. Proliferation rate fluctuated between 200 and 150 OD values at all concentrations of AVM 006**.** Remaining compounds AVM 005, AVM 009, AVM 012, AVM 013, and AVM 017 caused significant decline in proliferation of PBMC compared to the control indicating that these compounds have inhibitory effect on cell proliferation (Table 4.5). In Table, values over 1 indicate that tested compound stimulates PBMC to proliferate. Relative level of the cell proliferation was calculated by the following equation:

Relative Level of Cell Proliferation= Optical Density of the Sample / Optical Density of the Control

	Concentration					
Compounds	**0**	**0.1**	**1**	**100**	**500**	**1000**
AVM 003	1	0.4	1.32	0.64	0.73	0.97
AVM 004	1	1.15	1.42	1.23	1.15	1.48
AVM 005	1	0.44	0.54	0.46	0.41	0.362
AVM 006	1	0.76	0.97	0.73	0.67	0.78
AVM 007	1	0.9	0.83	1	0.86	0.95
AVM 009	1	0.42	0.69	0.44	0.36	0.61
AVM 012	1	0.1	0.23	0.16	0.28	0.26
AVM 013	1	0.13	0	0.25	0.45	0.27
AVM 017	1	0.14	0.62	0.3	0.33	0.44

Table 4.5. Summary of the effects of compounds AVM 003, AVM 004, AVM 005, AVM 006, AVM 007, AVM 009, AVM 012, AVM 013 and AVM 017 on cell proliferation (Results are the ratio of OD values of the compound to OD values of the control without compound). Data are the average of duplicate samples. The difference between OD values of duplicate samples was less than 3%.

5.5. AMES Mutagenicity Test

Mutagenic effects of compounds AVM 001, AVM 002, AVM 010, AVM 011, AVM 014, AVM 015 and AVM 019 were determined by AMES test. Compounds were dissolved in DMSO and each compound was examined at 0.01µM, 0.1µM, 1µM, 100µM, 1000µM and 3000µM concentration points. 100µl compound solute was mixed with 100µl bacterial culture. In this test, *Salmonella typhimurium* TA 100 and TA 102 strains were used. After 48 hours incubation at 37^{o}C, the number of revertant colonies with background lawn were counted and compared with the positive control (sodium azide and sunlight) and the negative control without any compounds. Because Sodium azide and sun light are known as mutagenic agents, they were served as a positive control in this current study. Since mutations is the main cause of the cancer chemotherapeutics do not have any mutagenic effects on the body. As a result, none of the newly synthesized compounds showed mutagenic effect on Salmonella TA 100 and TA 102. However, AVM 001 and AVM 002 showed anti-bacterial effect on Salmonella tester strains TA 100 and TA 102 (Figure 4.15, 4.16, Table 4.6 and 4.7); therefore, no colony was observed in the plate.

	Compound Concentrations							Mutagen
Compounds	**0**	**0.01**	**0.1**	**1**	**100**	**1000**	**3000**	**Sodium Azide**
AVM 001	93±2	0	0	0	0	0	0	1736±5
AVM 002	99±6	0	75±25	8±0	0	0	0	1168±19
AVM 010	82±7	66±6	60±5	62±13	65±5	69±14	76±4	1512±5
AVM 011	85±16	68±5	65±3	69±0	69±2	90±7	151±6	1540±4
AVM 014	85±10	55±7	61±12	58±5	62±1	60±3	74±4	740±0
AVM 015	77±28	57±4	49±0	63±20	54±40	59±3	68±10	878±50
AVM 019	63±4	63±12	63±40	67±21	67±0	72±21	80±23	882±11

Table 4.6. Mutagenic effects of the compounds AVM 001 AVM 001, AVM 002, AVM 010, AVM 011, AVM 014, AVM 015 and AVM 019 on TA 100 Salmonella strain and percent variation between duplicate samples.

	Compound Concentrations							Mutagen
Compounds	**0**	**0.01**	**0.1**	**1**	**100**	**1000**	**3000**	**Sun Light**
AVM 001	175±5	0	21±10	9±5	0	191±20	2±2	500±10
AVM 002	213±30	163±3	55±38	98±3	4±22	0	0	350±18
AVM 010	152±10	150±5	162±23	134±21	156±2	143±8	134±3	648±5
AVM 011	363±10	98±4	192±2	244±8	132±20	184±6	323±23	500±4
AVM 014	153±12	148±24	103±11	143±15	169±2	170±0	163±1	318±0
AVM 015	190±25	88±5	104±4	119±13	71±10	116±0	92±3	300±10
AVM 019	150±0	61±6	96±4	91±6	102±30	108±6	91±3	310±0

Table 4.7. Mutagenic effects of the compounds AVM 001 AVM 001, AVM 002, AVM 010, AVM 011, AVM 014, AVM 015 and AVM 019 on TA 102 Salmonella strain and percent variation between du plicate samples.

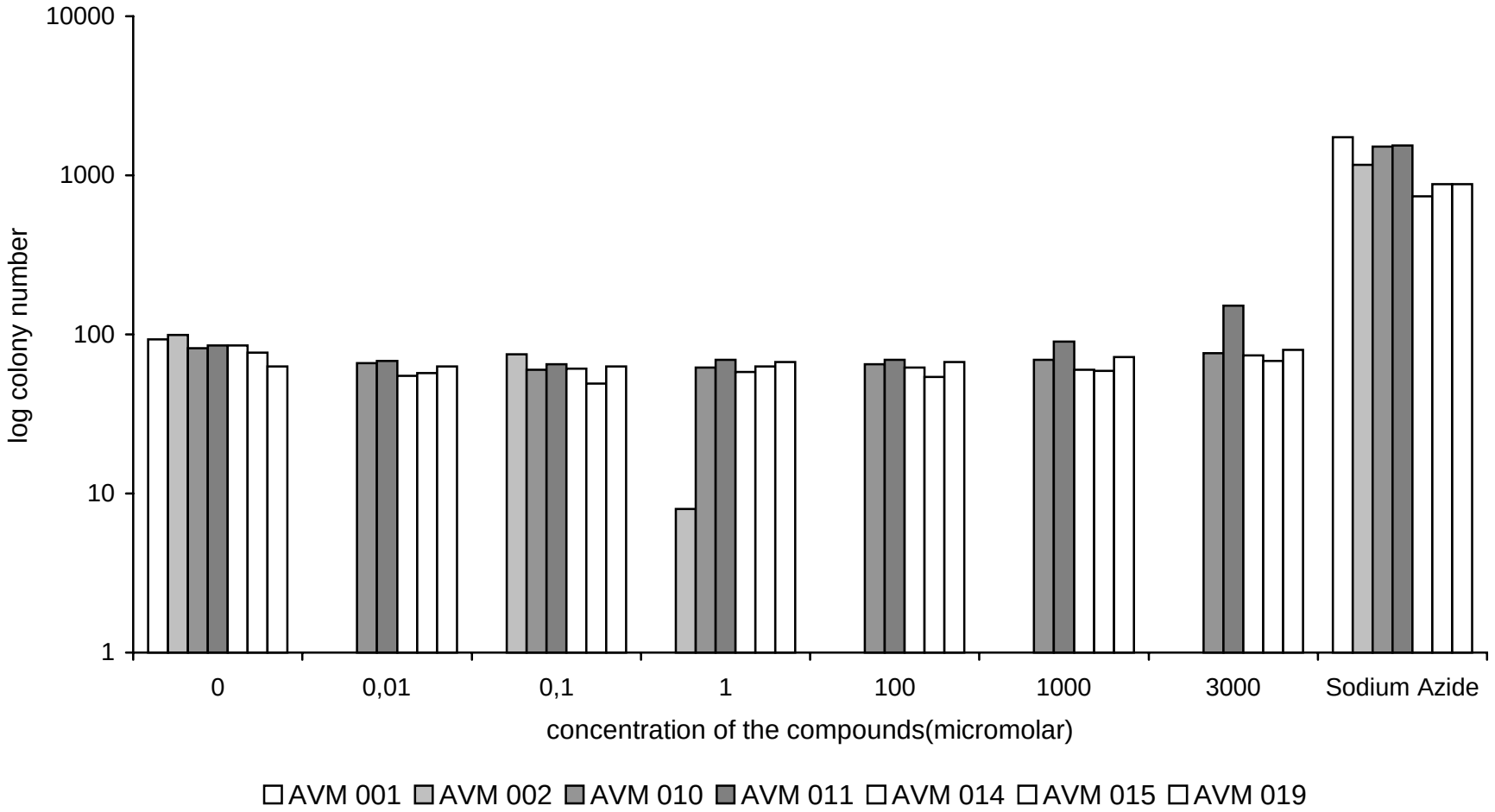


Figure 4.15. Mutagenic effects of the compounds AVM 001, AVM 001, AVM 002, AVM 010, AVM 011, AVM 014, AVM 015 and AVM 019 on TA 100 Salmonella strain (Results are the average of duplicate samples)

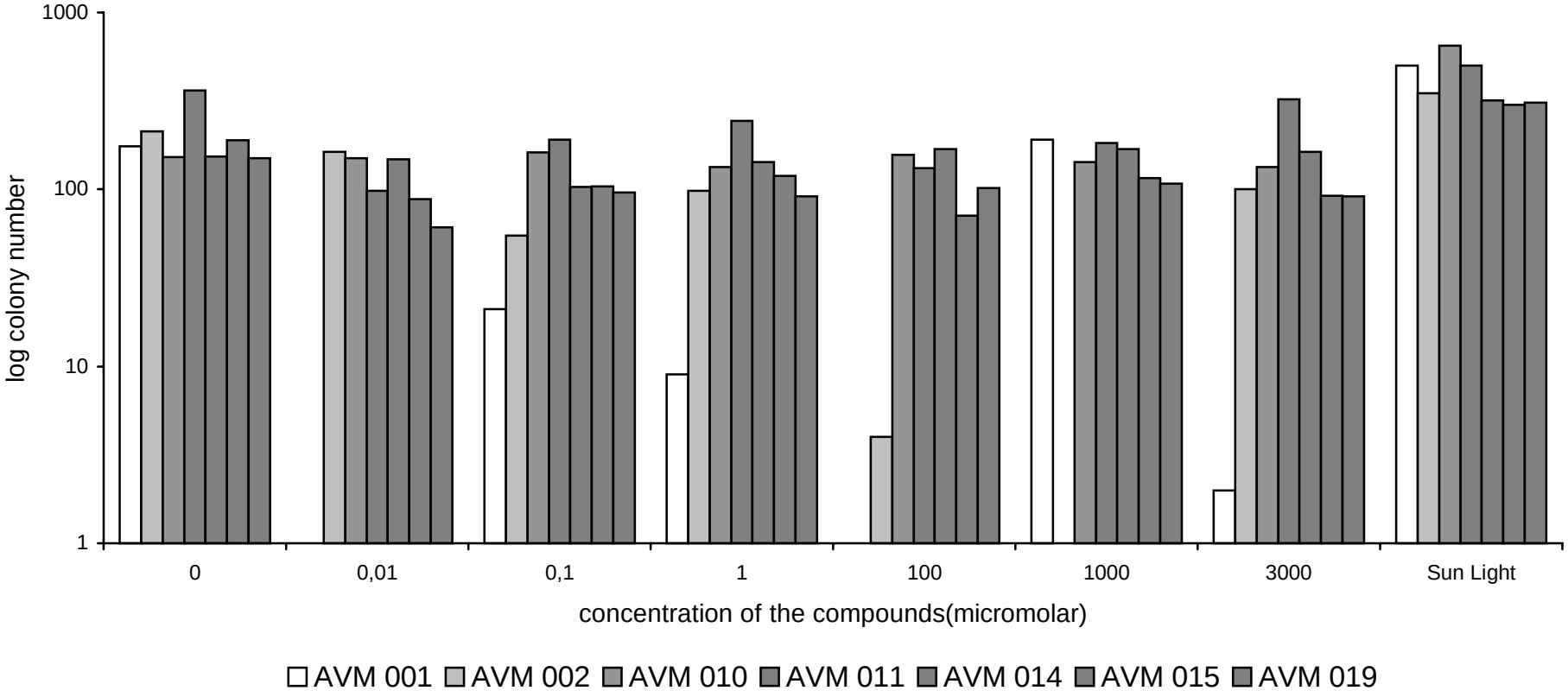
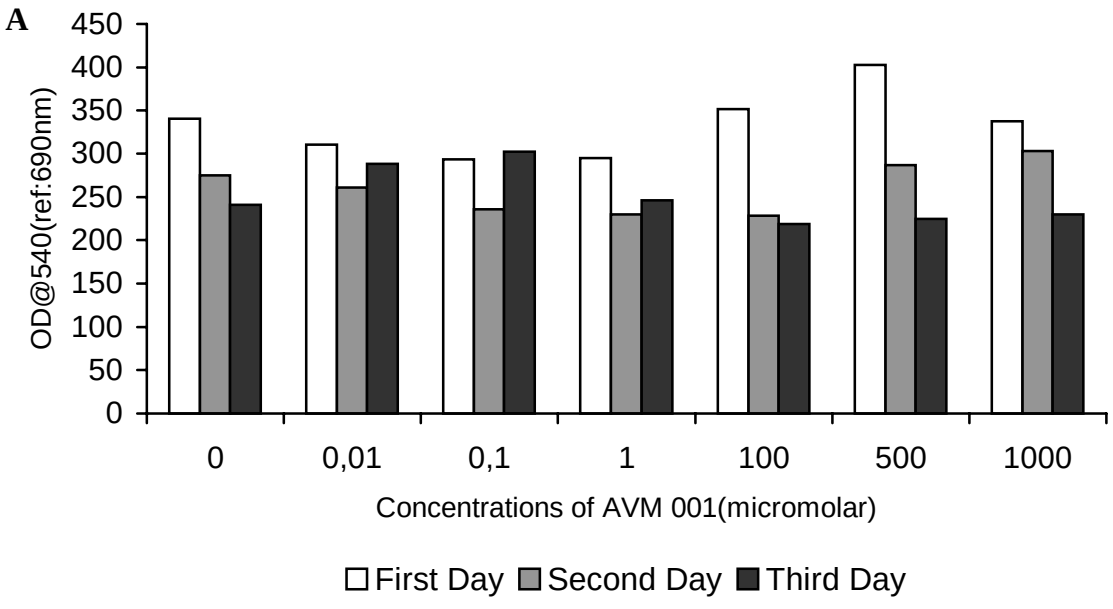


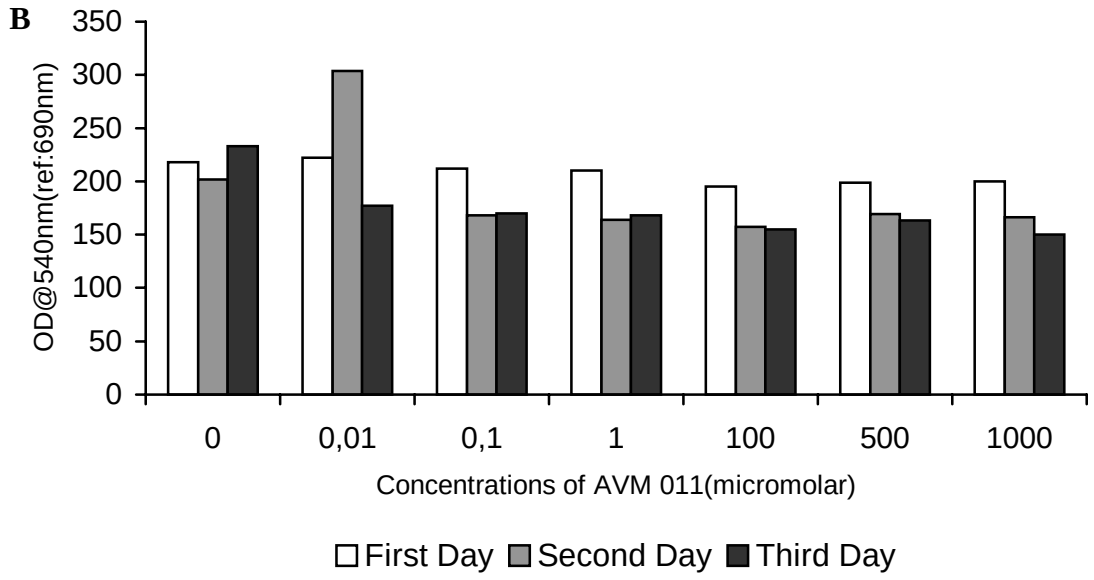
Figure 4.16. Mutagenic effects of the compounds AVM 001, AVM 001, AVM 002, AVM 010, AVM 011, AVM 014, AVM 015 and AVM 019 on TA 102 Salmonella strain (Results are the average of duplicate samples)

4.6.Activation Effects of The Compounds on PBMC

The activation effects of the compounds were examined by using MTT assay. Six different concentrations of the compounds (0.01µM, 0.1µM, 1µM, 100µM, 500µM, and 1000µM) were prepared and filter sterilized. In order to keep DMSO or ethanol concentration at the constant level per well, 100% pure solvent dilutions were prepared. After that, fixed amount of dissolved compounds were pipetted in to RPMI. Cells were treated with compounds at optimum cell culture conditions for 24, 48 and 72 hours. MTT was added 3 hours before the end of each incubation period. Formazan crystals formed by active cells were dissolved and absorbance of formed purple solutions directly represents the cellular activity. AVM 001 and AVM 011 did not show significant effects on the activation of PBMC when each day was compared with the controls (Figure 4.17.A and B).



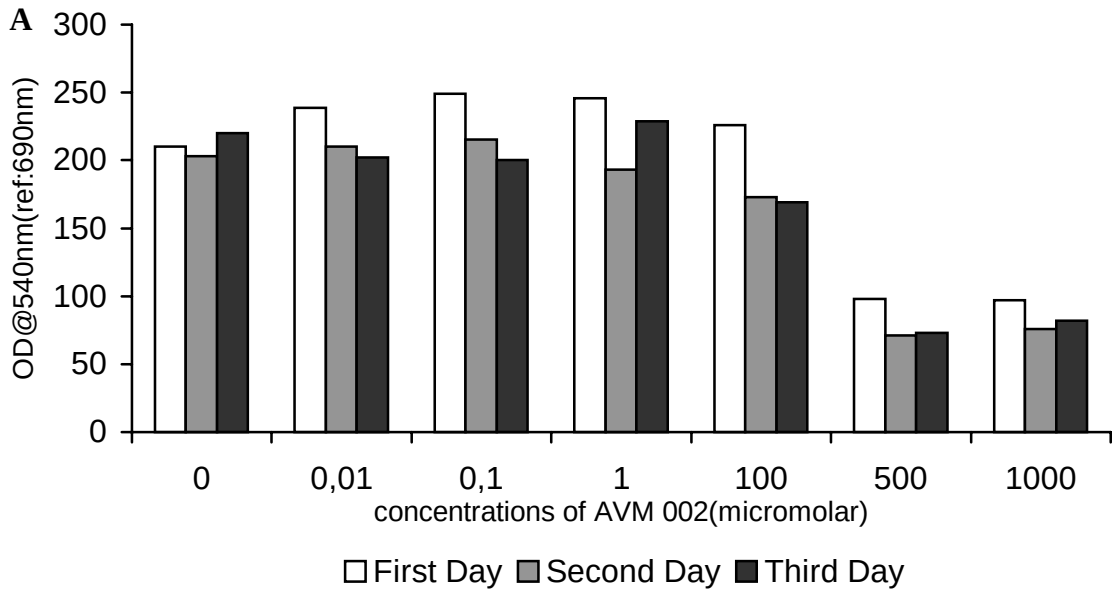
Activation Effect of AVM 001 on PBMC



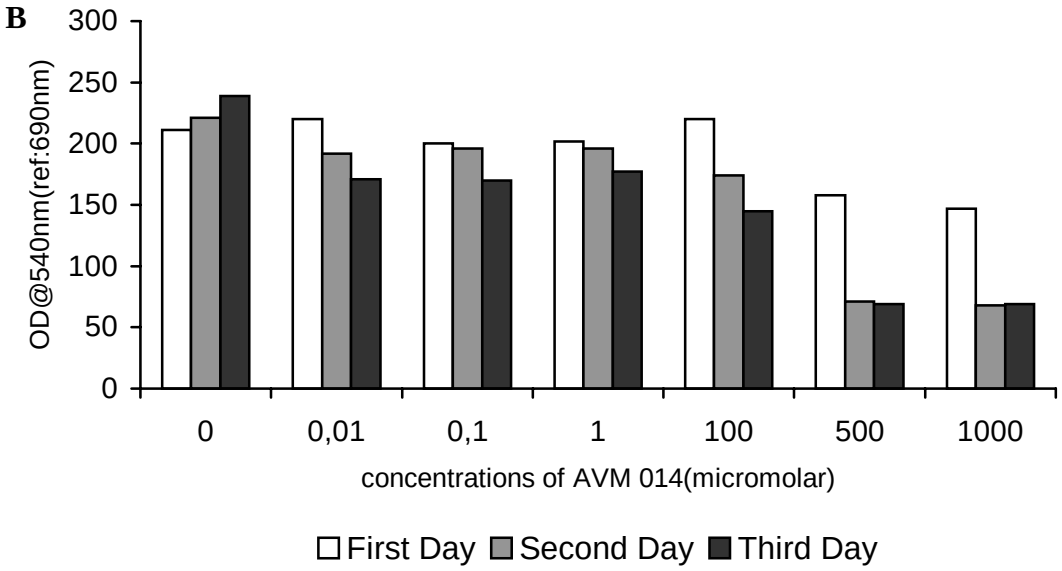
Activation Effect of AVM 011 on PBMC

Figure 4.17. Activation effects of AVM 001 (A) and AVM 011 (B) on PBMC. Data are the average of the separate experiments.

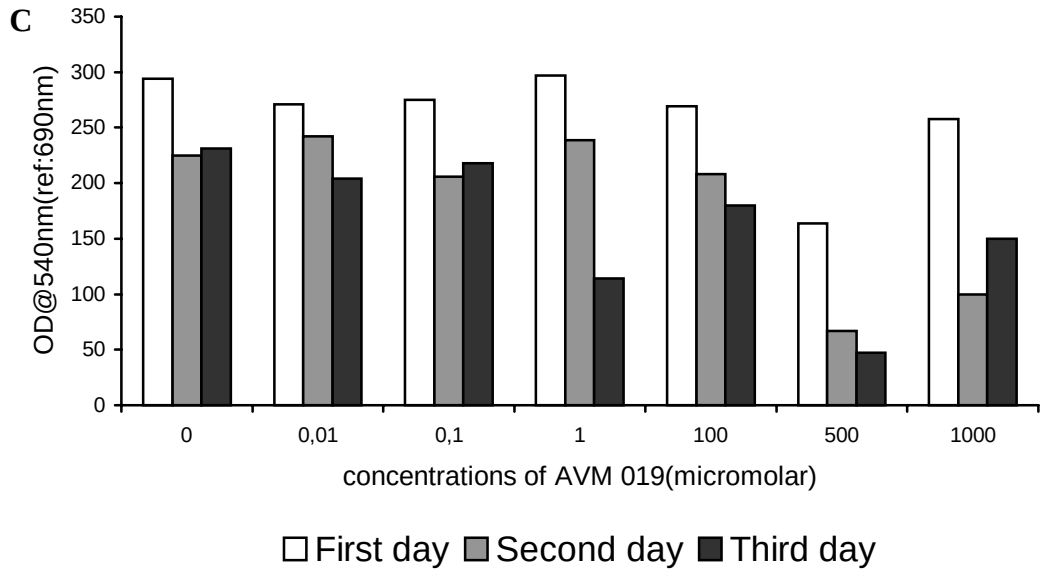
AVM 002, AVM 014, AVM 010 and AVM 019 caused decrease in cell activation especially at 500 μM and 1000 μM. AVM 014 and AVM 019 showed effect at the end of the 48 and 72 hours (Figure 4.18).



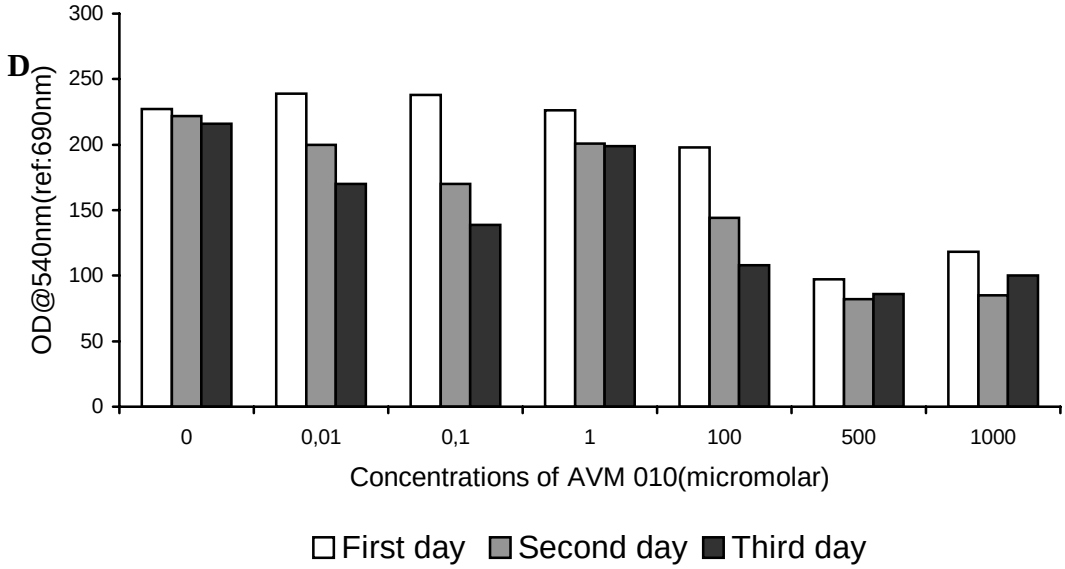
Activation Effect of AVM 002 on PBMC



Activation Effect of AVM 014 on PBMC



Activation Effect of AVM 019 on PBMC



Activation Effect of AVM 010 on PBMC

Figure 4.18. Activation Effect of AVM 002 (A), AVM 010 (D), AVM 014 (B) and AVM 019 (C) on PBMC. Data are the average of the separate experiments.

AVM 015 caused increase in cell activation of PBMC at 500 μM and 1000 μM concentration. The curve was similar with proliferation effect of the compound (Figure 4.19, Table 4.8).

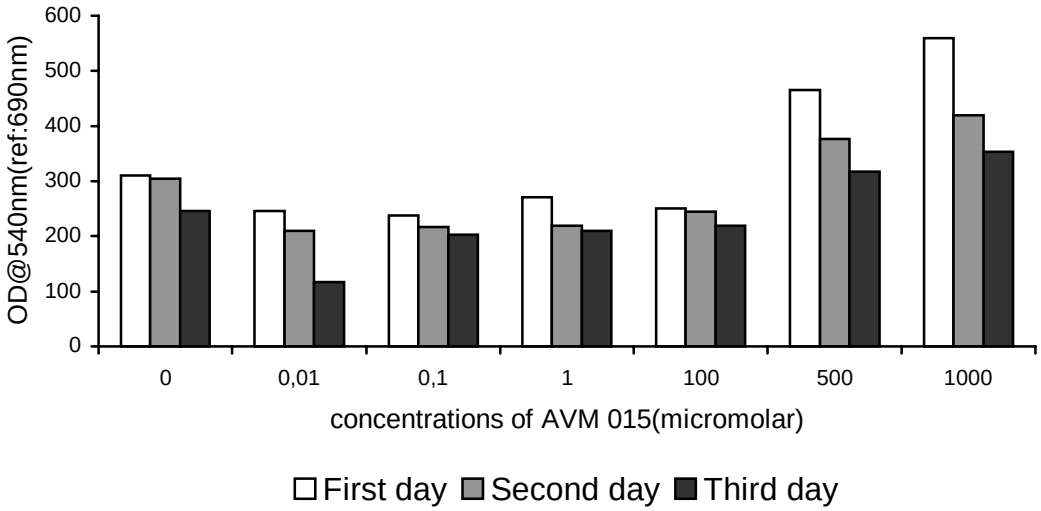


Figure 4.19. Activation Effect of AVM 015 on PBMC. Data are the average of the separate experiments.

		Concentrations						
Compounds	**Day**	**0**	**0.01**	**0.1**	**1**	**100**	**500**	**1000**
AVM 001	**1**	341±2	310±2	293±2	295±2	352±4	403±0	338±0
	2	275±1	261±10	236±1	230±2	228±0	287±3	303±4
	3	241±1	288±10	302±1	246±2	219±0	225±3	230±3
AVM 002	**1**	210±2	239±0	249±0	246±0	226±1	98±5	97±5
	2	203±3	210±3	215±0	193±5	173±3	71±3	76±6
	3	220±4	202±5	200±0	229±2	169±10	73±1	82±2
AVM 010	**1**	227±2	239±0	238±2	226±0	198±0	97±2	118±0
	2	222±2	200±3	170±4	201±8	144±2	82±5	85±0
	3	216±5	170±7	139±10	199±10	108±2	86±1	100±2
AVM 011	**1**	218±3	222±7	212±6	210±7	195±7	199±6	200±10
	2	202±3	304±3	168±3	164±10	157±2	169±4	166±6
	3	233±1	177±2	170±5	168±1	155±1	163±2	150±1
AVM 014	**1**	211±4	220±3	200±10	202±5	220±0	158±6	147±3
	2	221±1	192±7	196±5	196±7	174±9	71±2	68±1
	3	239±4	171±10	170±5	177±4	145±2	69±1	69±2
AVM 015	**1**	311±5	246±1	238±7	271±10	250±10	466±3	560±5
	2	305±4	210±5	217±10	219±3	244±10	377±0	419±2
	3	246±3	117±5	203±9	210±8	219±4	317±2	353±3
AVM 019	**1**	294±4	271±10	275±10	297±5	269±5	164±7	258±04
	2	225±4	242±1	206±1	239±2	208±2	67±3	100±0
	3	231±0	204±4	218±3	114±5	180±1	47±6	150±8

Table 4.8. Summary of the effects of the compounds AVM 001, AVM 002, AVM 010, AVM 011, AVM 014, AVM 015 and AVM 019 on cell activation. Results are average of OD values from separate experiments.

It can be concluded that AVM 001 and AVM 011 do not have any significant effect on the activation of the PBMC. AVM 002 and AVM 010 caused decline on the cell activity at 500μM and 1000μM concentrations at the end of three days. This decline can be caused from the accumulation of the compounds in cells. AVM 014 and AVM 019 caused decline in cell

activation especially at the end of second and third day and it can be caused from the metabolite accumulation. This is because; animal tests should be applied to find the reasons of this decline.

Chapter 5

CONCLUSIONS AND FUTURE EXPERIMENTS

5.1.Conclusion

In summary, HIV-1 RT inhibitory effects of the 19 compounds at the concentrations from 0.01 to 100 μM were investigated. For this aim, newly synthesized compounds were tested by using non-radioactive ELISA kit from Roche. Compounds AVM 001, AVM 002, AVM 010, AVM 011, AVM 014, AVM 015 and AVM 019 that showed RT inhibition in range of 0-100 μM were evaluated. In order to show blood cell effects, 3 parameters were investigated. These were viability, proliferation and activation of the Peripheral Blood Mononuclear Cells (PBMC). Effect of compounds on the viability of PBMC by trypan blue dye exclusion method, on the proliferation of PBMC by incorporation of bromodeoxyurudine to DNA in the proliferating cells and on the activation of PBMC by MTT test were examined. Determination of the mutagenic potentials of the compounds was performed by AMES test (*Salmonella typhimurium* reverse mutation test).

5-methyl-2-(phenoxymethyl) benzoxazole named as AVM 002 and AVM 014 (2-(p-fluorophenyl)-5-aminobenzoxazole) were found as the most promising compounds according to their RT inhibition effect when compared with inhibitory activity on RT of remaining compounds. IC50 (50 % inhibitory concentration, or concentration required to inhibit HIV-1 RT activity by 50 %) value of AVM 002 was found at 100μM concentrations. At the same concentration AVM 014 also showed 43% RT inhibition activity. Fifty percent cytotoxic concentration on PBMC (CC_{50}; 50% cytotoxic concentration, or concentration required to reduce viability of the host cells by 50 %) was observed at 500 μM. For other compounds, IC_{50} value was not found in the range of 0.01 μM-100 μM. At 100 μM concentration, compounds AVM 001, AVM 015, AVM 010, AVM 019, AVM 011 showed 38.4 %, 36 %, 34 %, 27 %, 25 % inhibitory effects on RT activity, respectively. Therefore, AVM 002 and AVM 014 can be evaluated by small modifications in compound structure to get higher RT inhibitory activity.

PBMC treated with AVM 001 at 1000μM concentration indicated 80 % viability at the end of 24 and 48 hours. Fourtyfive percent decline in the cell viability was

observed at the end of 72 hours only for the highest concentration and CC_{50} value was over 1000µM. AVM 002 and AVM 010 caused 50 % decrease in the viability of PBMC for 500 µM and 1000 µM compound concentration at the end of 24, 48 and 72 hours. CC_{50} of AVM 002 was equal to 500µM. AVM 010 was the most toxic one when compared with the dose-response curve of the other compounds, because at 1000 µM concentration 63 %, 67 % and 69 % decline in the cell viability were determined at the end of 24, 48 and 72 hours, respectively. CC_{50} of AVM 010 was found between 100µM-500µM concentrations. At the same concentration, AVM 014 also caused nearly 50 % decrease after 48 hours and 72 hours incubation. AVM 011, AVM 015 did not show any toxic effect on the viability of the PBMC at all the concentrations tested. In other words, CC_{50} values of AVM 011 and AVM 015 was over 1000µM concentrations for the cell viability. AVM 019 was toxic only at the end of third day at 1000µM concentration. As a result, the toxic effect of the compounds may be due to the compound structure, accumulation of compound in cells especially in the presence of high dose compound, or accumulation of the metabolite especially at the end of 48 and 72 hours. *In vivo* modeling of compound metabolism can explain the reasons of the toxicity and determine the dosage that should be used for HIV–1(+) individual.

Effects of the compounds on the proliferation of the PBMC varied. When compared with the control, AVM 001 caused 2.2-fold increase at 1 µM concentration and AVM 002 also caused 2.5-fold increase at the same concentration. In addition, IC_{50} value of AVM 002 (100µM) did not lead to any change on the proliferation of PBMC but decline in this parameter at 1000 µM concentration was observed for both AVM 001 and AVM 002. AVM 014 resulted in a linear decline until the highest concentration, 1000 µM at which 3 fold decline was obtained. AVM 010 and AVM 011 also caused 50 % decrease on this parameter when compared with the control. While AVM 019 was found to stimulate the cells to proliferate, AVM 015 caused quite different proliferation profile. AVM 015 at 0.01µM concentration gave rise to 75% decrease in cell proliferation, compared to the control; when the concentration increased, cell proliferation rate rose. Inhibition of cell proliferation can lead to immune suppression and make the body more susceptible to infections. Since AIDS patients have less than 200 CD4+ cells, additional decrease in cell proliferation depending on the usage of drugs can cause increase in the probability of death due to the opportunistic infections especially at the last period of HIV infection. Therefore, AVM 010 and AVM 011 are

not useful as an anti-HIV chemotherapeutics. Since AVM 019 caused increase in cell proliferation, it seems to be safe according to this parameter. Other compounds require additional tests with infected cell lines with HIV-1. In addition, AVM 004 can be evaluated as an immunostimulant.

In addition, activation of PBMC in the presence of the compounds was evaluated and closely associated results were obtained with proliferation experiments. At the end of first 24 hours, AVM 001 caused a little increase at 100 µM and 500 µM concentrations but a decrease in the activation of PBMC was observed at the end of 72 hours. PBMC treated with AVM 011 resulted in little decline at the end of 48 and 72 hours when compared with the controls of each days. While 500 µM and 1000 µM concentrations of AVM 002 and AVM 014 caused around 60 % decline, other concentrations did not affect activation of PBMC. For the same parameter 500 µM AVM 019 caused 60 % decrease at the end of 48 and 72 hours but the cell activation at 1000 µM concentration was higher than the activation of 500 µM AVM 019 treated wells. AVM 010 caused a gradual decrease depending on the increase in compound concentration. On the contrary, increased concentrations of AVM 015 caused elevation in cell activation and 500µM and 1000 µM concentrations resulted 25 % and 40 % increase, respectively. Therefore, it can be concluded that AVM 001 and AVM 011 do not have any significant effect on the activation of the PBMC. Decline on the cell activity at 500µM and 1000µM concentrations of AVM 002 and AVM 010 can be resulted from the accumulation of the compounds in cells. Decline in the cell activation especially at the end of second and third day of AVM 014 and AVM 019 exposure can be caused from the metabolite accumulation. This is because; animal tests should be applied to find the reasons of this decline.

According to results of AMES reverse mutation test, none of the compounds have mutagenic effects on *Salmonella typhimurium* TA 100 and TA 102 strains when they were compared to the positive controls (sodium azide and sun light) and the negative control.

As a result, especially AVM 002 and AVM 014 are the most promising compounds in regard to RT inhibition. A slight modification in their chemical structure of these compounds may give rise to significant increase in the inhibitory effects of them on RT enzyme. Such changes in their structure will allow us to investigate these compounds at the second stage level of drug development research. In other words, this

current study revealed the inhibitory effects of compounds on RT and completed the studies on the first stage of drug discovery.

5.2. Future Experiments

i. Structures of the compounds should be modified in order to increase RT inhibition effects and decrease cytotoxicity
ii. Second generation compounds should be evaluated *in vitro*
iii. Computer programs can be used to find fitness of compounds to hydrophobic pocket of HIV-1 RT enzyme in order to support *in vitro* results
iv. Second generation compounds can be evaluated with HIV infected cell lines to find utility for HIV (+) individuals
v. All compounds and also second generations should be assayed on resistant strains
vi. In order to find half life of compounds, *in vivo* metabolism should be worked with animals

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