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Section of General Microbiology and Virology & Microbial Biotechnologies

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XXIV Cycle

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Synthesis and anti-retroviral activity of novel 1,2-benzisothiazol-3(*2H*)-one benzenesulfonamides targeting the HIV nucleocapsid protein 7

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ABSTRACT

The biological activity of new 1,2-benzisothiazol-3(2H)-one benzenesulfonamides is described. In cell-based assays the lead compound **6** inhibits multiplication, but not entry into target cells, of HIV-1, HIV-2 and HIV-1 variants carrying clinically relevant mutations to non-nucleoside, nucleoside and protease inhibitors. In enzyme assays **6** fails to inhibit HIV-1 reverse transcriptase and integrase. Genome sequencing of HIV-1 mutants selected for resistance to **6** shows no mutations in both *pol* and *env* genes. On the contrary, two mutations map in the *gag* region coding for the nucleocapsid (NC) protein p7 (NCp7), which is involved in early and late key processes of retrovirus multiplication.

Overall, the above results suggest that NCp7 is the target of title 1,2-benzisothiazol-3(2H)-one benzenesulfonamides.

Interestingly, **6** also shows concentration-dependent virucidal activity against cell-free HIV-1 and HIV-2. Therefore, title compounds represent a new class of antiretroviral agents with intriguing spectrum and mode of action.

1.0 Origin of HIV

Human immunodeficiency viruses type-1 (HIV-1)[1,2] and type-2 (HIV-2)[3] have been reported as the primary cause of the Acquired Immunodeficiency Syndrome (AIDS).

They had been transmitted to humans by Central and West Africa primates, respectively. The animal virus most closely related to HIV-1 is the simian immunodeficiency virus (SIV), thus far identified in members of the Pan troglodytes troglodytes chimpanzees;[4] the one most closely related to HIV-2 is the SIV identified in Sooty mangabey monkeys.[5]

The discovery of HIV as the cause of AIDS has been one of the major scientific achievements of the last century. It started, in 1981, with the recognition of the abrupt incidence rise of two diseases occurring in previously healthy persons: pneumonia from Pneumocystis carinii and Kaposi's sarcoma. Within the next 2 years, the causative agent had been identified as the human immunodeficiency virus (HIV).[6] In 1983 Barré-Sinoussi published a paper describing the isolation of a putative new human retrovirus from the lymph gland of a patient suffering from persistent generalized lymphadenopathy,[1] which is regarded as a precursor condition of AIDS. Simultaneously, Gallo and his colleagues reported the isolation of a human retrovirus, known as human T-cell leukemia virus (HTLV), from AIDS patients and advocated a role for this retrovirus in the pathogenesis of AIDS.[2] These reports have been pioneering in the discovery and characterization of HIV.[7]

Both HIV-1 and HIV-2 have been classified as members of the genus *Lentivirus*, within the *Retroviridae* family.[8] *Lentiviruses*, which are responsible for long-duration illnesses with a long incubation period, are single-stranded, positive-sense, enveloped RNA viruses.

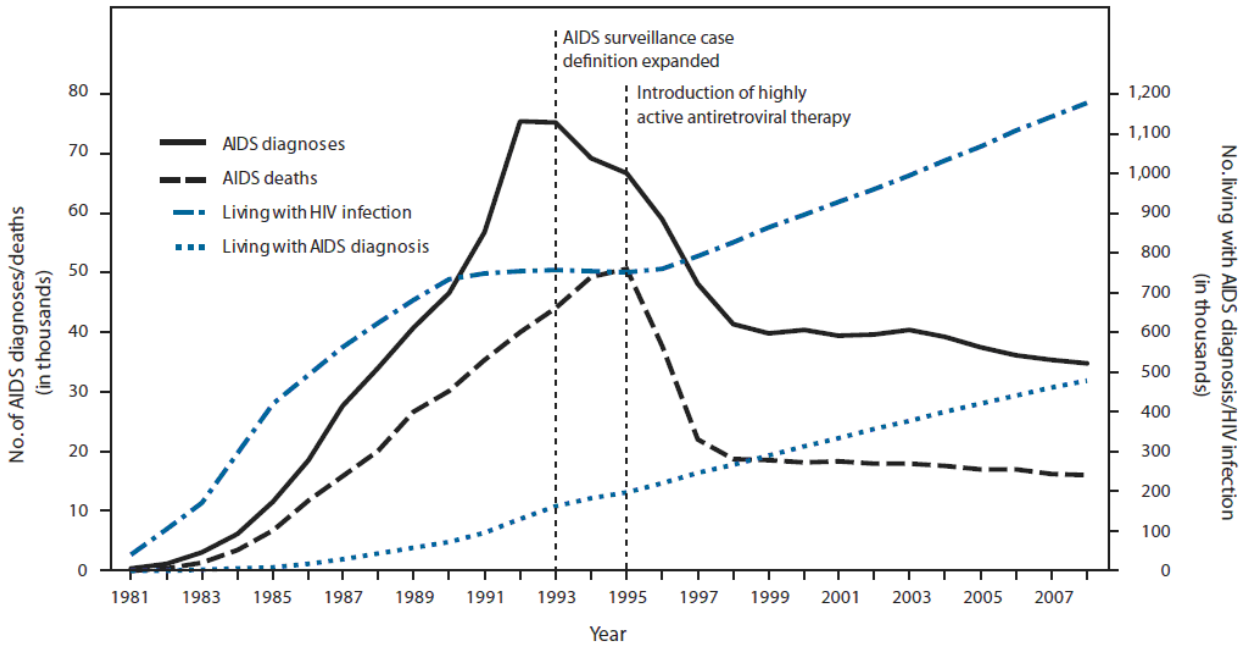
HIV infections lead to AIDS through progressive destruction of the immune system and degeneration of the central and peripheral nervous systems. Being the first cause of death in Africa, and the fourth leading cause of death worldwide, AIDS is a major health problem.[9] Therefore, despite the considerable progress made in the treatment of HIV infections/AIDS, there is continued interest in the development of new therapeutic agents directed against traditional and new targets in the HIV replication cycle.[10]

Both HIV-1 and HIV-2 genomes carry equivalent structural and accessory genes. Exceptions are the *vpu* gene, unique to HIV-1, and the *vpx* gene, present only in HIV-2.[11] *Vpu* is a 16 kDa (81 aa) protein with at least two different biological functions: degradation of CD4 in the endoplasmic reticulum, and enhancement of virion release from the plasma membrane of infected cells. *Vpx* is a 12 kDa protein whose function has been recently reported to play a role in nuclear import.

2.0 Epidemiology

To characterize trends in the HIV infection/AIDS in the United States during 1981-2008, CDC analyzed data from the National Surveillance System.[12] In the 14 years since 1981, sharp increases had been reported in the number of new AIDS diagnoses and deaths among persons aged ≥13 years, reaching peaks of 75,457 and 50,628 in 1992 and 1995, respectively (Figure 1). Following the introduction of the highly active anti-retroviral therapy (HAART), AIDS diagnoses and deaths declined substantially from 1996 to 1998 and remained stable from 1999 to 2008, at an average of 38,279 AIDS diagnoses and 17,489 deaths per year (Figure 1). At the end of 2008, an estimated 1,178,350 persons aged ≥13 years were living with the HIV infection, including 236,400 individuals (20.1%) whose infections had not been diagnosed (see Fig. 1).

FIGURE 1. Estimated number of AIDS diagnoses and deaths and estimated number of persons living with AIDS diagnosis* and living with diagnosed or undiagnosed HIV infection† among persons aged ≥13 years — United States, 1981–2008



The global HIV/AIDS pandemic is one of the deadliest epidemics of modern times. In 2007 a total of 2.0 million men, women and children died of AIDS, worldwide. Unfortunately, in the next future the death toll will remain high, due to the fact that 33 million individuals are currently infected and about 2.7 million new HIV infections occur each year. Most of the currently and newly infected individuals are likely to die of AIDS eventually, despite the increasing availability of anti-retroviral treatments. A cumulative total of 24 million people died from AIDS between 1980 and 2007, and by 2030 this total is projected to reach 75 million. Despite the rapid spread of the disease during the

1980s and 1990s, the epidemic has reached a major turning point in recent years, as the rate of new infections peaked and began to decline (Figure 2).[12]

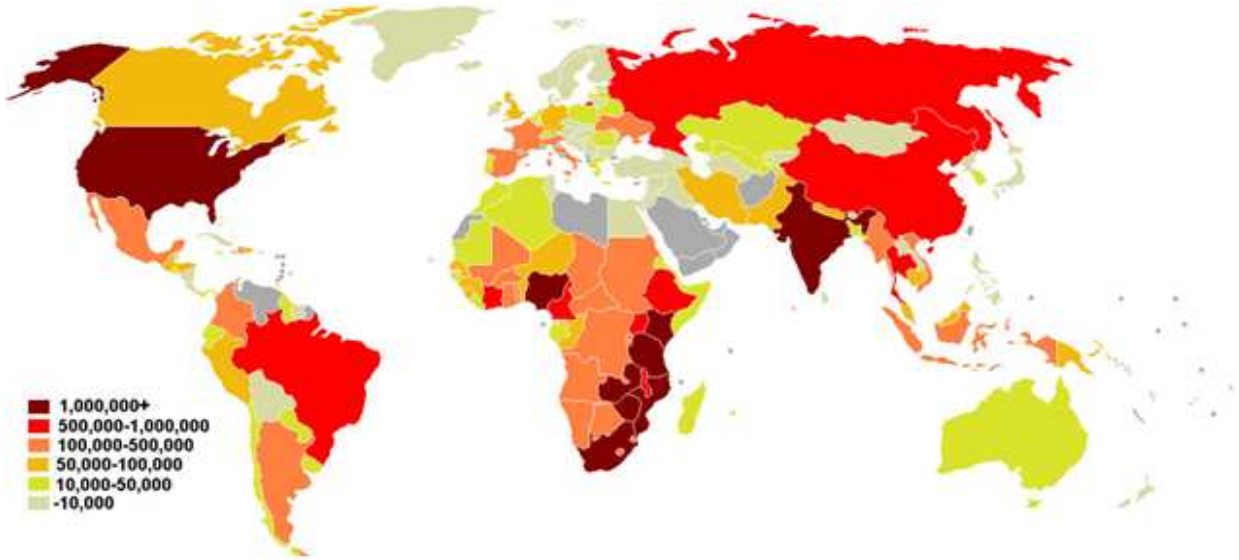


Figure 2. Estimates of people living with AIDS

3.0 HIV transmission

3.1 Sexual transmission

Most HIV infections worldwide are transmitted sexually. Transmission occurs via contact between sexual secretions of one partner with the rectal, genital or oral mucous membranes of the other. Sexual transmission probabilities depend on the infectiousness of the HIV-infected partner and the susceptibility of the uninfected partner. Infectiousness and susceptibility depend on behavioural, biological, genetic, and immunological risk factors of the host and the virus. Infectivity seems to vary during the course of illness and is not constant between individuals.[13]

Sexually transmitted infections show increased risk of transmission and infection when disruption of the normal epithelial barrier exist due to genital ulceration and/or micro-ulceration, and when accumulation of pools of HIV-susceptible or HIV-infected cells (lymphocytes and macrophages) occur in semen and vaginal secretions. Epidemiological studies from sub-Saharan Africa, Europe and North America have suggested that there is approximately a four times greater risk of becoming infected with HIV in the presence of genital ulcers, such as those caused by syphilis. There is also a significant, though less increased, risk in the presence of sexually transmitted infections, such as gonorrhoea, Chlamydia infection and trichomoniasis, which cause local accumulation of

lymphocytes and macrophages.[14] However, an undetectable plasma viral load does not necessarily indicate a low viral load in seminal fluid or genital secretions.[15]

During a sexual act, only male or female condoms can reduce the chances of infection with HIV and other sexually transmitted diseases. The National Institutes of Health (NIH) defines "condom effectiveness" as the level of protection against HIV and other sexually transmitted diseases (STDs) when condoms are used consistently and correctly. The use of condom, during every act of intercourse among heterosexual couples in which one partner is infected with HIV, reduces the risk of HIV transmission from men to women, and vice versa. Analyses of condom effectiveness studies, and their comparison with no condom use, allowed to estimate that consistent condom use may result in an overall 87 % reduction in the risk of HIV transmission.[16]

3.2 Non-sexual transmission

Mother to Child

A pregnant mother who has HIV can transmit the virus to the fetus or baby. The infant can become infected *in utero*, during delivery or through feeding on infected breast milk. In the US, mothers to be are screened for HIV. If the mother is infected, anti-retroviral therapy can start. Treatment may also include C-section, instead of vaginal birth, and avoiding breast feeding.[17]

Blood Transfusion

This was a more common way to contract HIV in the 1970s and 1980s. Since 1985, testing for HIV in all donated blood, blood products and organs has been systematically carried out in the US.[18]

Households

Although HIV has been transmitted among family members in a household setting, this type of transmission is very rare and may result from contact between skin/mucous membranes and infected blood. To prevent even such rare occurrences, precautions should be taken in all settings, home include, to prevent exposure to the blood of persons who are HIV-infected, or at risk for HIV infection, or whose infection and risk status are unknown.

For example:

- Gloves should be worn during contact with blood or other body fluids that could possibly contain blood, such as urine, feces, or vomit.
- Cuts, sores or breaks, on both the care giver's and patient's exposed skin, should be covered with bandages.

- Hands and other parts of the body should be washed immediately after contact with blood or other body fluids, and surfaces soiled with blood should be disinfected appropriately.
- Practices that increase the probability of blood contact, such as sharing of razors and toothbrushes, should be avoided.
- Needles and other sharp instruments should be used only when medically necessary, and should be handled according to recommendations for health-care settings.[19]

Kissing

Casual contact through closed-mouth or "social" kissing is not a risk for HIV transmission. Because of the potential for contact with blood during "French" or open-mouth kissing, CDC recommends against engaging in this activity with a person known to be infected. However, the risk of acquiring HIV during open-mouth kissing is believed to be very low. CDC has investigated only one case of HIV infection that may be attributed to contact with blood during open-mouth kissing.[20]

Biting

HIV transmission by biting is possible but extremely unlikely. In order for transmission to take place, there would need to be both exposure to blood and a route into the body for that blood. Just two case reports have documented infection via this route. However, there have been many more case reports where a bite by somebody with HIV did not result in HIV infection.[20]

Fighting

Bleeding onto intact skin during a fight poses no HIV exposure or transmission risk. If HIV-infected blood comes into contact with broken skin, then HIV transmission is possible but still unlikely. The small number of case reports documenting HIV transmission via this route involved a significant amount of blood from the HIV-positive person, as well as large open wounds in the other person's skin.[21]

Wounding with a needle

The risk of HIV transmission from exposure to an HIV-infected needle outside healthcare settings has not been studied. However, despite media reports that often suggest that the risk is great, there is not a single recorded case anywhere in the world of someone being infected with HIV through an attack with a needle or by accidental wounding with a needle outside health care settings.

The risk of HIV infection following a needle stick injury is low. Risk of infection may be higher if the needle was used in an HIV-positive person's vein or artery, if the HIV-positive person has a high viral load, or if the needle stick injury is deep[22]

4.0 Virion structure

Infections with *Lentiviruses* typically show a chronic course of disease, a long period of clinical latency, persistent viral replication and involvement of the central nervous system. Examples are Visna infections in sheep, SIV infections in monkeys or feline immunodeficiency virus (FIV) infections in cats.

HIV-1 and HIV-2 resemble each other strikingly. However, they differ with regard to the molecular weight of their proteins, as well as having differences in their accessory genes. Using electron microscopy, HIV-2 is genetically more related to the SIV (simian immunodeficiency virus) than HIV-1, and it is likely that they were introduced into human population from monkey population. Both HIV-1 and HIV-2 replicate in CD4+ T cells and are regarded as pathogenic in infected persons, although the immune deficiency may be less severe in HIV-2-infected individuals.[23]

HIV particles (Figure 3) have a diameter of 100 nm and are encircled by an envelope consisting of a host cell–derived lipid bilayer fitted with virus-encoded glycoproteins. Each virion contains 72 glycoprotein complexes, which are integrated into this lipid membrane, and are each composed of an external glycoprotein gp120 and a transmembrane protein gp41. The viral surface glycoprotein is encoded in the *env* gene region of the HIV genome; in fact, the primary *env* gene product gp160 is proteolytically processed into the two non-covalently associated subunits gpl20 and gp41.[24]

The surface subunit gp120 determines viral tropism through interaction with the primary cellular receptor CD4 and particular chemokine receptors. The trans-membrane subunit gp41 mediates direct fusion of the viral envelope with the cellular membrane. The matrix protein p17 is anchored to the inside of the viral envelope. The p24 core (or capsid) contains two copies of the HIV RNA, which is complexed with the p7 nucleoprotein (NCp7) and the p66 reverse transcriptase (RT). The viral particle contains all the enzymatic equipment that is necessary for replication: RT, integrase (p32) and protease (p11).

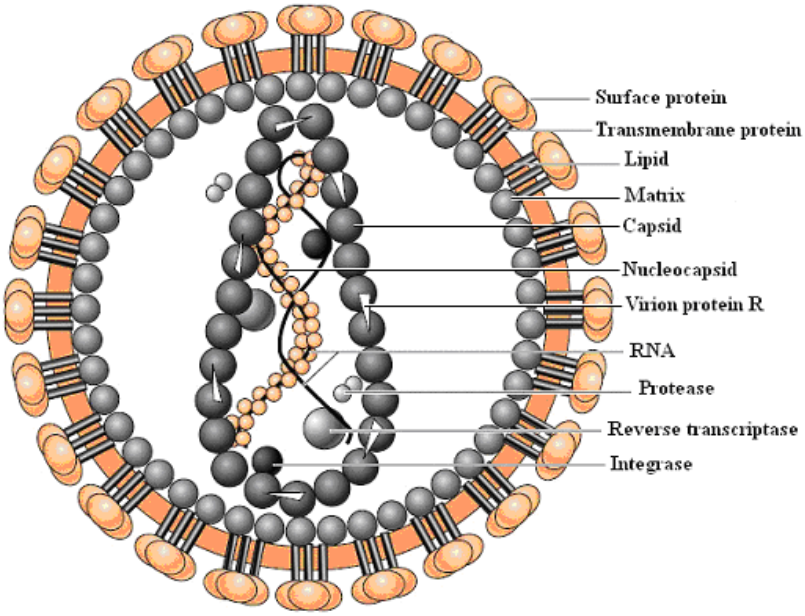


Figure 3. HIV structure

5.0 Genome organization

The replication of Retroviruses depends on three genes: *gag*, *pol* and *env* ("group-specific antigen", "polymerase" and "envelope", respectively). The classical structural scheme of a retroviral genome (Figure 4) is: 5' LTR - *gag* - *pol* - *env* - LTR 3'. The LTR (long terminal repeat) regions represent the terminal parts of the viral genome (not encoding for viral proteins) which, after integration, connect the viral and the host cell DNAs. The *gag* gene codes for the nucleocapsid proteins; *pol* codes for crucial enzymes, such as protease, reverse transcriptase and integrase; *env* codes for the envelope glycoproteins.

In addition, HIV genomes contain six genes (*vif*, *vpr*, *tat*, *rev*, *vpu* (HIV-1) / *vpx* (HIV-2) and *nef*) that contribute to its genetic complexity. *Vif*, *vpr*, *vpu* and *nef* have been classified as accessory genes, as they seem to be not absolutely required for replication in vitro.

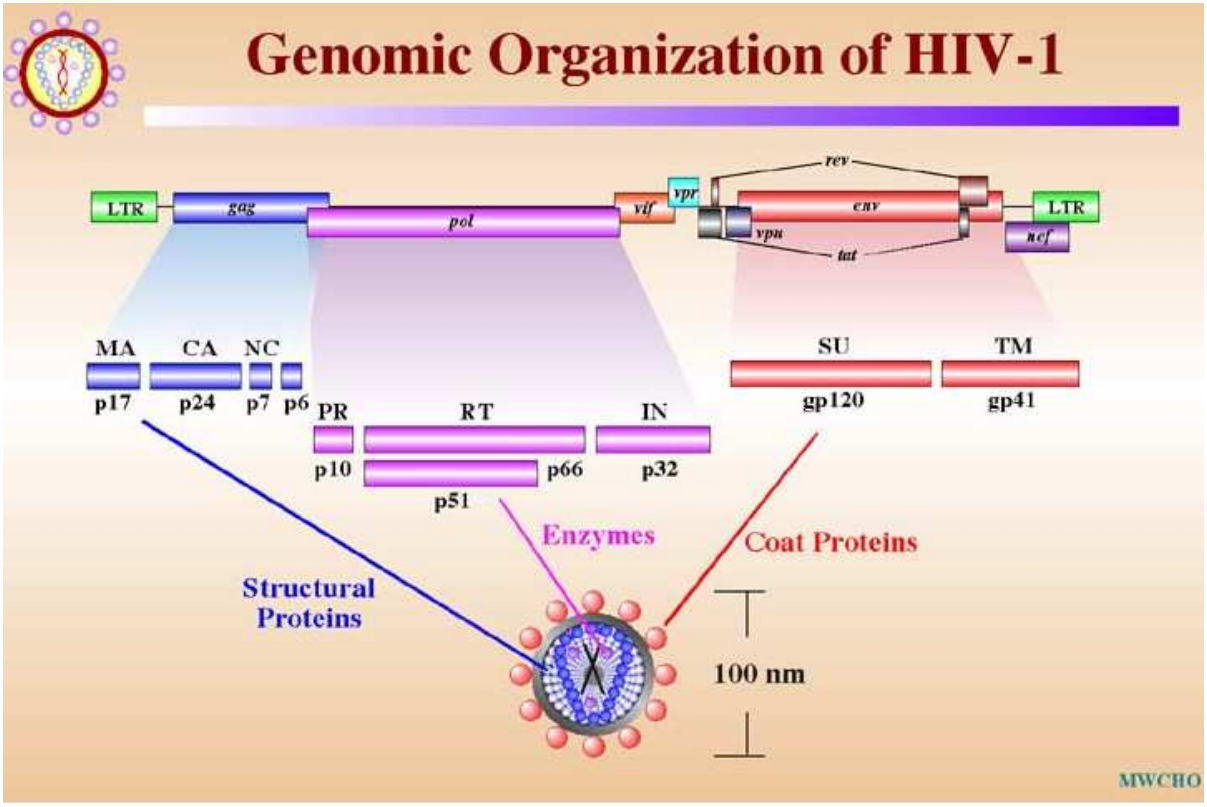


Figure 4.

Both regulation and function of accessory genes and their proteins have been studied and characterized in detail during the past few years. The regulatory genes *tat* and *rev*, and the accessory gene *nef*, are all expressed early in the viral replication cycle.[25]

Tat is a potent transcriptional activator of the LTR promoter region and is essential for viral replication in almost all in vitro culture systems. Tat and rev stimulate transcription of the proviral DNA into RNA, promote elongation of the latter, enhance its transportation from the nucleus to the cytoplasm and are essential for translation. Rev is a nuclear export factor that is important for switching from the early expression of regulatory proteins to the late expression of structural proteins. Nef has been shown[26] to have a number of functions that may induce down regulation of CD4 from the surface of HIV-1-infected cells, which may represent an important escape mechanism for the virus to evade an attack mediated by cytotoxic CD8+ T-cells and to avoid recognition by CD4+ T-cells. Nef may also interfere with T-cell activation by binding to various proteins that are involved in intracellular signal transduction pathways. In SIV-infected rhesus macaques, an intact *nef* gene is essential for a high rate of virus production and progression of the disease. Vpr seems to be essential for viral replication in non-dividing cells, such as macrophages. It may stimulate the LTR in addition to a variety of cellular and viral promoters, and is important for the transport of the viral pre-integration complex to the nucleus. Vpu is important for the virus "budding" process, and

mutations in the *vpu* gene are associated with persistence of viral particles at the host cell surface. Vpu is also involved when CD4-gp160 complexes are degraded within the endoplasmic reticulum and therefore allows recycling of gp160 for the formation of new virions.[27,28]

6.0 Replication cycle

The life cycle of retroviruses (Figure 5) is arbitrarily divided into two distinct phases: the early phase refers to the steps of infection from cell-binding to integration of the viral cDNA into the cell genome; the late phase begins with the expression of viral genes and continues through the release and maturation of progeny virions.[29]

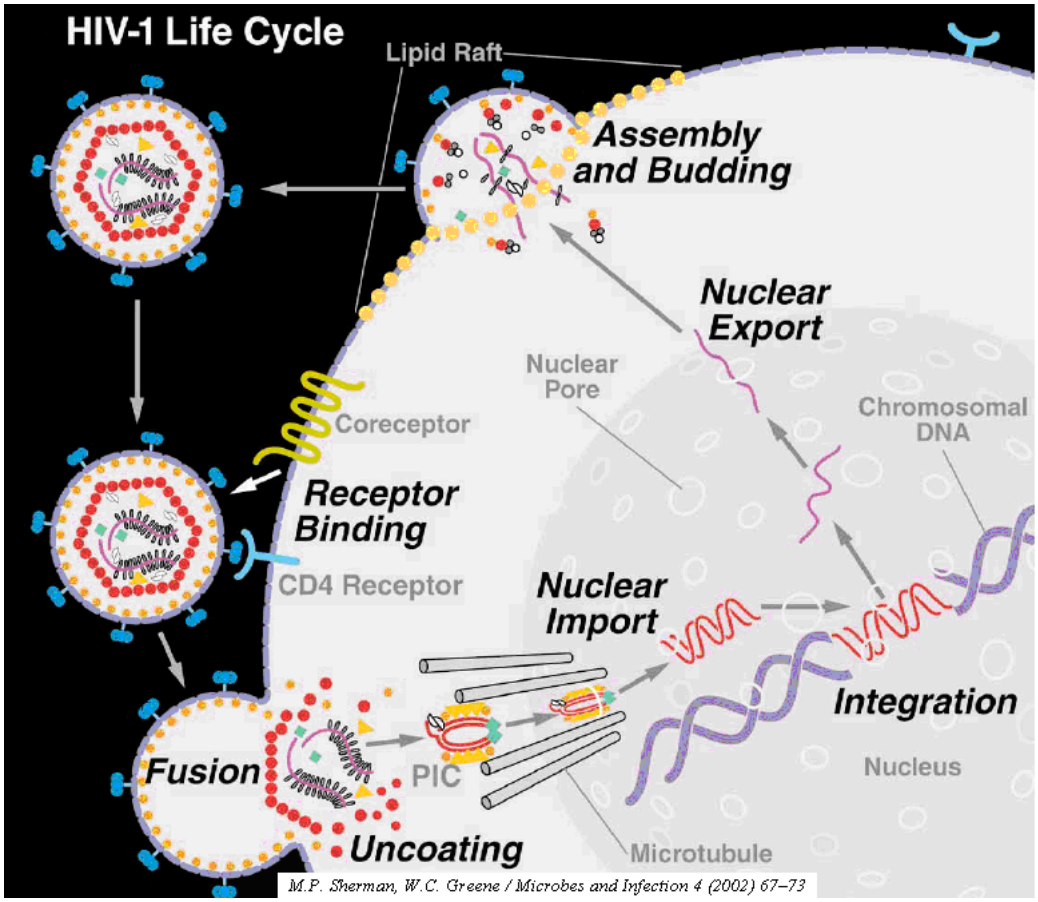


Figure 5. HIV life cycle

6.1 Binding

Attachment of the virus particle to appropriate receptor molecules in the host-cell plasma membrane is considered to be the initial step in all virus infections. CD4 receptors and co-receptors (CCR5 or CXCR4) are involved in the initial step of HIV replication cycle. All $CD4^+$ cell lines, including the

human T-cell lines MT-2 and MT-4 , are susceptible to HIV infection and develop rapid cytopathic effects.[24]

The binding of virions to the cell surface has been attributed to a variety of cell-surface molecules, including heparan sulphate proteoglycan, LFA-1 and nucleolin. Heparan sulfates (HS) are highly sulfated polysaccharides, widely expressed on the surface of cells and which have been shown to be utilized as cell surface attachment factors by numerous viruses and bacteria. However, although the involvement of HS in HIV-1 attachment has been widely documented, its exact role remains somewhat controversial. It is interesting to note that even retrovirus-like particles lacking envelope proteins are able to bind cells via interactions with HS, confirming that the initial attachment of retroviruses to cells is, at least to a certain extent, env-independent. However, it is known that env-independent binding of HIV leads to the endocytosis of particles, which is a dead end with respect to cell infection.[29]

6.2 Entry

The HIV envelope glycoproteins gp120 and gp41 are non-covalently associated and form trimers on the surface of the virus particle. HIV entry into target cells is initiated by the consecutive interaction of the soluble unit gp120 with CD4 and a co-receptor, whereas virus-cell membrane fusion is mediated by the trans-membrane unit gp41.

Binding of the initial receptor CD4 induces changes in gp120 conformation that allow high-affinity interaction with the co-receptor, CCR5 or CXCR4, as well as formation of a gp41 pre-hairpin intermediate. Engagement of co-receptor promotes additional conformational changes in gp41 that create an energetically stable six helix bundle coincident with the fusion of viral and cell membranes.[30]

HIV particles enter the cells in two alternative ways (Figure 6). In the fusion mode, the viral envelope fuses with the plasma membrane and accomplishes cell penetration and uncoating simultaneously. HIV particles may also enter the cell by endocytosis of adsorbed virus into a vesicle. HIV uptake by endocytosis seems to occur mainly in coated vesicles, but some virus particles may be taken up by other means, such as phagocytosis. The above two entry modes exhibit almost the same frequency, although the area containing the fused cell membrane and the virus envelope is difficult to evidenciate. Both entry mechanisms have been found in lymphocytes and macrophages.[31]

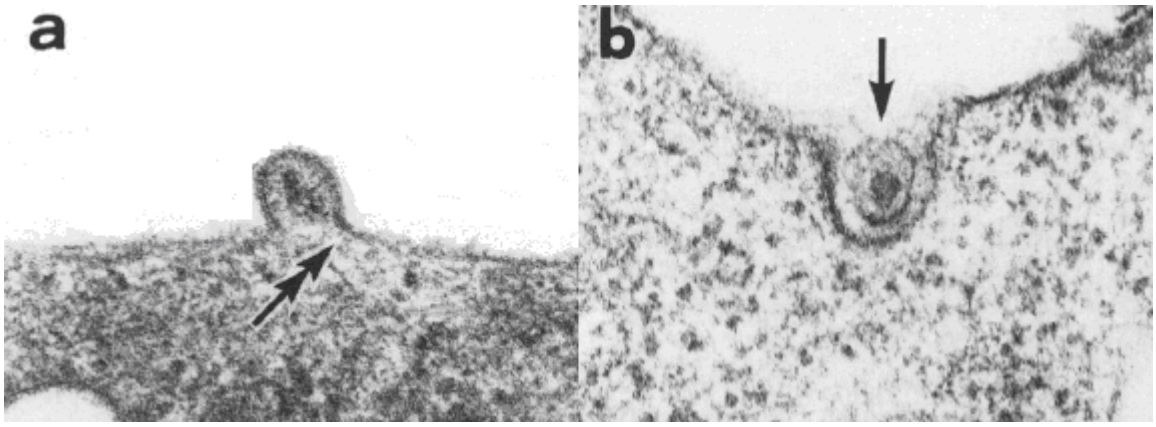


Figure 6. The two entry modes of HIV: [a]fusion; [b]endocytosis

6.3 Uncoating and reverse transcription

Reverse transcription usually occurs after release of viral cores into the cytoplasm of target cells. Immediately after release into the cytoplasm, the viral core undergoes a partial and progressive disassembly, known as uncoating. It leads to the generation of sub-viral particles called reverse-transcription complexes (RTCs) and pre-integration complexes (PICs). Initiation of reverse transcription is coupled with uncoating of the viral core. It should be noted that the distinction between RTCs and PICs is somewhat arbitrary since uncoating is believed to occur progressively. Thus, RTCs contain incompletely reverse-transcribed genomes, whereas PICs are integration-competent complexes containing protease (PR), reverse-transcriptase (RT), integrase (IN) and vpr. Capsid (CA), nucleocapsid (NC) and matrix (MA) proteins are released soon after infection and only trace amounts of them are found in PICs.[29]

6.4 Trafficking of incoming viruses through the cytoplasm

After penetration into host cells, HIV viruses have to reach the nucleus to replicate. The cytoplasm, containing a high protein concentration in addition to organelles and the cytoskeleton, constitutes a medium in which incoming particles cannot rely on simple passive diffusion to move. Consequently, viruses have evolved numerous and specific mechanisms to hijack cellular machinery, and in particular the cytoskeleton, to facilitate their spread within the infected cells.

Some studies have revealed that the use of specific drugs altering the integrity of the cytoskeleton can interfere with the HIV cycle, either by directly affecting the intracellular trafficking of incoming viruses or by interfering with other steps of the early phase of infection, such as reverse transcription. Indeed, it has been shown that an intact actin cytoskeleton is essential for efficient reverse transcription of HIV. Additional reports have described specific interactions between

retroviral proteins and cytoskeleton components. For example, HIV IN and NC have been shown to interact with yeast microtubule-associated proteins and actin, respectively, although the precise role of such interactions in intracellular trafficking of incoming viruses remains to be elucidated.[29]

6.5 Nuclear entry

The retroviral life cycle requires integration of the viral DNA into the host cell genome to form the so-called provirus. To this end, the reverse-transcribed DNA, associated in PICs with viral proteins, must enter the nucleus. PICs from most retroviruses are unable to enter intact nuclei and must therefore "wait" for the breakdown of the nuclear membrane occurring during mitosis. Consequently, retroviruses (such as MLV) are dependent on the cell cycle and cannot replicate in non-dividing cells. In contrast, *Lentiviruses* (such as HIV) are able to productively infect non-dividing cells, such as macrophages or quiescent T lymphocytes, indicating that PICs are able to actively cross the nuclear membrane.

HIV PICs, composed of the double-stranded linear DNA associated with the viral proteins RT, IN, MA and Vpr, have an estimated Stokes diameter of 56 nm. Since the central channel of the nuclear pore has a maximum diameter of 25 nm and the pore is known to be able to transport macromolecules up to 39 nm, HIV has developed a strategy to achieve the challenge of passing through these structures. Nuclear pore complexes (NPCs) are large supra-molecular protein structures that span the nuclear membrane and protrude into both cytoplasm and nucleoplasm. Signal-mediated nuclear import involves the interaction of nuclear localization signals (NLS) (which are typically short stretches of amino acids) in proteins with nucleo-cytoplasmic shuttling receptors belonging to the karyopherin β family, also known as importins. Four different viral components have been identified to contribute to the nuclear import of HIV. Among the constituents that are believed to form the PIC, IN, MA, Vpr and the viral DNA are suspected to play a significant role in nuclear import, either directly or indirectly, although the exact function of each remains to be fully understood.[29]

Integrase (IN) has been considered to be the main mediator of HIV nuclear translocation for some time, but its exact implication is now being re-evaluated. This viral protein, which harbours a non-classical NLS, seems to be both necessary and sufficient to promote the nuclear accumulation of viral PICs. The nature of the pathway used by this NLS is not known, but, interestingly, the nuclear import function of IN was found to be essential for productive infection of both non-dividing and dividing cells.[29]

Matrix protein (MA) is the second protein which has been proposed to possess karyophilic properties. It has been found to contain a classical basic NLS in its N-terminal region, responsible for targeting the PIC into the nucleus. Viral strains with alterations in the NLS replicate normally in dividing cells, indicating that the mutations affect neither viral assembly nor any other essential MA function. However, NLS mutants can not efficiently replicate in non-dividing cells (macrophages or T cells). These findings led to the hypothesis that the HIV NLS might be critical for infection of macrophages.[29]

Vpr is perhaps the most controversial protein with respect to its role in PIC import. This HIV protein is a 96-aminoacid polypeptide that is packaged into progeny virions through its interaction with the C-terminal p6 Gag domain of the p55 Gag precursor protein. In recent studies it has been shown that Vpr alters the structure of the nuclear lamina, leading to nuclear herniations that intermittently rupture. These ruptures in the nuclear envelope may provide a freely accessible portal for uptake of the large HIV PICs.[31]

DNA flap is the final product of HIV-1 reverse transcription and is a linear DNA molecule bearing in its centre a stable 99 nucleotide–long plus-strand overlap. The HIV gene transfer vectors lacking the central DNA flap exhibit a strong nuclear import defect. The insertion of the DNA flap sequence into such a defective vector increases its transduction efficiency by complementing its nuclear import defect to wild-type levels. The central DNA flap could act as a viral determinant for initiation of the crawling of the HIV DNA filament through the nuclear pore. This could be achieved through direct interaction of the DNA flap with components of the pore or, alternatively, through interaction with cellular or viral proteins that shuttle between the cytoplasm and the nucleus of the host cell and could drag the HIV genome into the nucleus.[32]

6.6 Integration

To replicate, a retrovirus must integrate a DNA copy of its RNA genome (provirus) into a chromosome of the host cell. Viral gene activity varies in different chromosomal locations. Efficient transcription of viral genes, following integration in active transcription units, can facilitate productive host infection.

The initial steps of DNA integration are catalyzed by the viral integrase enzyme (IN). First, IN cleaves two nucleotides from the 3’ end of each viral DNA strand, exposing a 3’-OH group. Then, it simultaneously breaks the host DNA and joins it to the viral 3’ end (strand transfer) by a single-step trans-esterification reaction. The third step is thought to involve host DNA repair proteins to remove the two nucleotide overhang and to fill in the DNA gaps.[33]

6.7 Viral transcription

Transcription from the provirus is regulated by the activity of the HIV promoter, the long terminal repeat (LTR) found at the 5' end of the DNA. The LTR possesses binding sites for numerous cellular transcription factors, including Sp1, NFΠB, AP-1, and NF-AT. Given that these factors are responsible for T cell activation, it is not surprising that the latter promotes viral expression. Once integrated, the HIV provirus may lie dormant within a cell for a long time. However, once activated, the cell treats the HIV gene in much the same way as human genes. First, it converts it into a mRNA (using cellular enzymes). Then, the mRNA is transported outside the nucleus and is used as a blueprint for producing new HIV proteins and enzymes. The proviral expression is also dependent on local chromatin environment. In the absence of premature termination, expression from the provirus results in the generation of a "full length" RNA. This non-spliced transcript serves as a messenger for several HIV structural proteins (gag–pol genes), as well as an RNA genome to be incorporated into newly synthesized HIV particles. As a rule, common to host and retroviral gene expression, association of the RNA transcripts with an assortment of proteins, including splicing enzymes, results in removal of introns and efficient delivery of mature messages to the cytosol. The full-length HIV transcripts also contain a variety of splicing donor and acceptor sites. This feature of HIV permits the encoding of various proteins in overlapping genes (within the same segment of DNA), and permits a temporal separation of gene expression. In the infected cell, the earliest RNA generated becomes fully spliced by the cellular splicing machinery.[34]

6.8 Viral assembly and budding

The assembly of the virus takes place on the cell surface in the form of budding. Before assembly, the virus glycoproteins, the internal proteins and the RNA must reach the plasma membrane from their sites of synthesis. The nucleoproteins and the other virus proteins (located within the virus particle) stay in the cytoplasm after synthesis, diffusing to the cell surface and binding to the cytoplasmic face of the plasma membrane. Findings by immunoelectron microscopy with p24-MoAbs suggest that HIV-1 Gag is produced in membrane-bound structures and transported to the cell surface by the cytoskeleton. Near the cell membrane, Gag precusor proteins will be cleaved to mature forms, such as p17 and p24 (Figure 7a). Virus particles with mature cores are then released from the cell surface. The budding regions are seen to react intensively with anti-HIV gpl20 antibodies from an AIDS patient's serum, but the rest of the cell surface is less reactive with these antibodies. The area of budding appears (Figure 7b) to be strictly limited to surface regions containing viral proteins.[34]

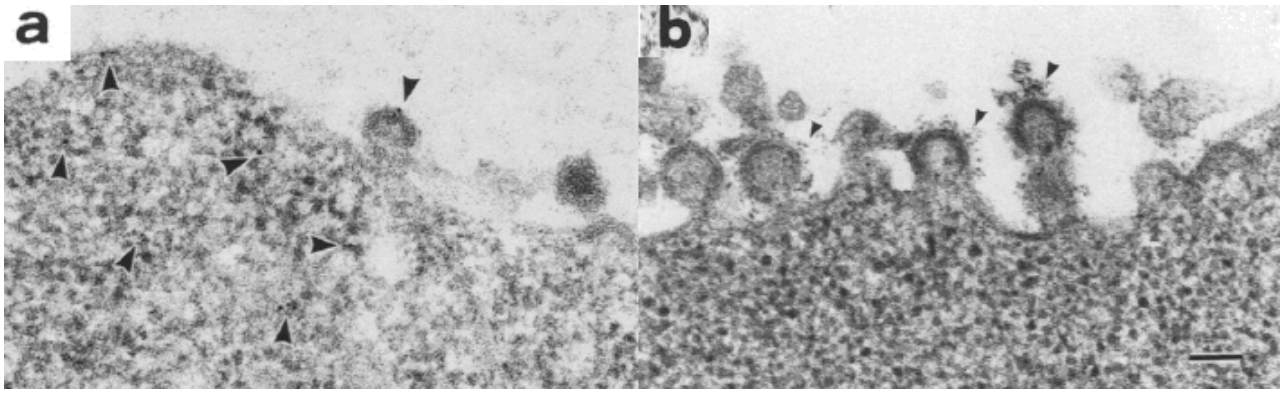


Figure 7. Viral assembly and budding
[a]Immuno-gold labelling (5 nm gold particles) of post-embedded HIV-1-infected cells with anti-HIV-1 Gag p24 monoclonal antibody. A viral bud, the cell membrane and some parts of the cytoplasm (arrowheads) are labelled. Scale bar indicates 100 nm.
[b]Immuno-electron micrograph of budding with ferritin labelling (arrowheads). Many crescent-shaped electron-dense layers were formed at the cell membrane of a cell infected with HIV-1. Scale bar indicates 100 nm.

7.0 Antiretroviral Drugs

Numerous viral targets susceptible of selective inhibition have been identified. These include: *env*-encoded CD4-binding gp120 and fusion gp41 proteins; *pol*-encoded enzymes essential for HIV replication: reverse transcriptase (RT), integrase (IN) and protease (PR); regulatory proteins, such as TAT and REV, and *gag*-encoded proteins, such as the nucleocapsid p7 (NCp7).[35,36,37,38]

The first FDA-approved drugs for the treatment of HIV infection/AIDS have successfully exploited two essential enzymes encoded by the *pol* gene: RT and PR. More recently, three FDA-approved drugs target the third *pol*-encoded enzyme, IN, and various cellular and viral proteins (CD4, CCR5, CXCR4, gp120 and gp41) involved in the entry process of HIV.[39-40]

The aim of antiretroviral treatment is to keep the amount of HIV in the body at a low level.[41] This stops any weakening of the immune system and allows it to recover from any damage that HIV might have caused already.

Currently, there are 31 compounds which have been formally approved (by the US Food and Drug Administration) for the treatment of HIV infections (AIDS), and their widespread use has resulted in a significant decrease in HIV-related morbidity and mortality (FDA, 2011). They belong to the following classes.

Entry inhibitors[42,43] are a class of antiretroviral drugs that block virus from entring host by:

1. Bind to the CCR5 co-receptor and prevent its interaction with the gp120 protein.
2. Block the gp41 protein conformational changes necessary for the fusion process.

Nucleoside reverse transcriptase inhibitors (NRTIs) are the first class of antiretroviral drugs developed. With respect to physiological counterparts, they present modifications at the 3' position of the sugar moiety that affect their capability to form diester linkages in the elongation process. In order to be incorporated into the viral DNA, NRTIs must be activated in the cell by the addition of three phosphate groups to their deoxyribose moiety, to form NRTI triphosphates. This phosphorylation step is carried out by cellular kinase enzymes. Being analogues of the naturally occurring deoxynucleotides needed to synthesize the viral DNA, NRTIs compete with the natural deoxynucleotides for incorporation into the growing viral DNA chain. Thus, when an NRTI is incorporated, viral DNA synthesis is halted, a process known as chain termination.[44]

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) block the reverse transcriptase by binding at an allosteric site present in the proximity of the catalytic site. Upon binding, these compounds cause a conformational distorsion of the surrounding domains that affects the proper functioning of the catalytic site needed to carry out the process of DNA synthesis. NNRTIs are therefore classified as non-competitive inhibitors of reverse transcriptase.[45]

Integrase Inhibitors (INs) target the enzyme responsible for the integration of the viral genetic material into the human DNA, a crucial step in the replication cycle of HIV. Raltegravir is the first drug approved in the class of antiretroviral drugs called integrase inhibitors.[46]

Protease Inhibitors (PIs) are substrate analogues of the HIV enzyme involved in the processing of viral proteins: the protease. Once bound to its active site, any further activity of the enzyme is prevented. This causes an halt of the virion maturation process leading to formation of complete and infectious particles.[47]

The different classes of antiretroviral drugs are listed below:

Inhibitors	Approval Date	Manufacturer Name
EIs (Entry Inhibitors)		
Enfuvirtide	2003	Hoffmann-La Roche Trimeris
Maraviroc	2007	Pfizer

Inhibitors	Approval Date	Manufacturer Name

NRTIs (Nucleoside Reverse Transcriptase Inhibitors)		
Zidovudine, Azidothymidine	1987	GlaxoSmithKline
Didanosine, dideoxyinosine	1991	Bristol Myers-Squibb
Stavudine	1994	Bristol Myers-Squibb
Lamivudine, 3TC	1995	GlaxoSmithKline
Lamivudine and Zidovudine	1997	GlaxoSmithKline
Abacavir sulphate	1998	GlaxoSmithKline
Abacavir, Zidovudine, Lamivudine	2000	GlaxoSmithKline
Enteric Coated Didanosine	2000	Bristol Myers-Squibb
Tenofovir	2001	Gilead Sciences
Emtricitabine, FTC	2003	Gilead Sciences
Abacavir and Lamivudine	2004	GlaxoSmithKline
Tenofovir, Emtricitabine	2004	Gilead Sciences

Inhibitors	Approval Date	Manufacturer Name
NNRTIs (Non-nucleoside Reverse Transcriptase Inhibitors)		
Nevirapine	1996	Boehringer Ingelheim
Delavirdine	1997	Pfizer
Efavirenz	1998	Bristol Myers-Squibb
Etravirine	2008	Tibotec
Rilpivirine	2011	Tibotec

Inhibitors	Approval Date	Manufacturer Name
InIs (Integrase inhibitors)		
Raltegravir	2007	Merck

Inhibitors	Approval Date	Manufacturer Name
PIs (Protease Inhibitors)		
Saquinavir mesylate	1995	Hoffmann-La Roche
Ritonavir	1996	Abbott Laboratories
Indinavir	1996	Merck
Nelfinavir mesylate	1997	Agouron Pharmaceuticals
Amprenavir	1999	GlaxoSmithKline
Lopinavir and Ritonavir	2000	Abbott Laboratories
Fosamprenavir Calcium	2003	GlaxoSmithKline
Atazanavir sulphate	2003	Bristol Myers-Squibb
Tipranavir	2005	Boehringer Ingelheim
Darunavir	2006	Tibotec

8.0 Highly active antiretroviral therapy (HAART)

The intrinsic high genetic variability of HIV, related to the error-prone nature of RT, which continuously generates mutants capable to overcome inhibition by all known drugs, calls for the combination of antiretroviral drugs with different modes of action. The aim is to achieve maximum suppression of viral multiplication and to provide the most effective barrier to the emergence of drug-resistant mutants. As a matter of fact, the possibility to target RT with two different classes of inhibitors, NRTIs and NNRTIs, has allowed to prove the efficacy of the so called highly active antiretroviral therapy (HAART), initially based on triple combinations of drugs belonging to NRTIs, NNRTIs and PRIs. It is likely that this therapeutic approach will become more and more successful as the number of classes, and of drugs within each class, will increase.[48,49,50]

HAART has dramatically altered the treatment and life expectancy of individuals who are infected with HIV. When renal aspects of treatment for HIV infection were first reviewed in the Journal of the American Society of Nephrology 15 years ago, zidovudine was the only antiretroviral agent available, the life expectancy of HIV-infected patients was often measured in months, and there was debate as to whether patients with HIV infection should be started on dialysis. Now, more than 20 antiretroviral drugs and drug combinations are available, HIV viral load can be suppressed to undetectable levels and life expectancy of HIV-infected individuals is measured in decades.

9.0 Drug resistance

One of the major challenges to be faced in the clinic is the insurgence of resistance to the different classes of antiretroviral drugs.

This phenomenon, which is the inevitable consequence of incomplete suppression of the HIV replication, accompanied by the rapid turnover of the HIV-1 RNA and by its genetic variability, leads to the production of HIV variants with decreased drug susceptibility. Moreover, many patients with a primary infection demonstrate some degree of resistance to at least one of the currently available classes of drugs.[51]

Appearance of drug-resistant virus strains under the selective pressure of anti-HIV chemotherapy rapidly occurs as a consequence of the low fidelity of the RT-catalyzed DNA polymerisation reaction and the massive viral turnover. Resistance-associated mutations appear in the RT of virus strains that are under selective pressure of both NRTls and NNRTls. Sometime, these mutations cause cross-resistance to other drugs belonging to the same class, a phenomenon particularly true for NNRTIs. Usually, however, mutations do not affect the potency of the other available drug classes. Moreover, specific mutations have been identified that restore the initial drug sensitivity, when concomitantly present in addition to those impairing the susceptibility to a given drug.

Combination therapy has proven to be able to markedly suppress virus replication (and subsequent appearance of drug resistance) for a relatively long time period. However, in a number of cases, multiple drug combination therapy results in the appearance of a different mutation spectrum than is expected to emerge under mono-therapy. All available information argues for the use of a rational combination of different anti-HIV inhibitors with different resistance spectra to suppress virus replication efficiently and to delay the emergence of drug-resistant virus as long as possible. However, it also indicates that there is a strong need for additional drugs to further optimize and improve the efficacy of long-term HIV treatment.[52]

The limited duration of the anti-HIV response that occurs in most patients treated with protease inhibitor monotherapy is associated with the appearance of drug-resistant virus

In general, initial single amino acid mutations yield only a slight change in drug sensitivity. However, secondary mutations accumulate and can lead to high-level drug resistance. Prolonged treatment with one protease inhibitor can result in the emergence of virus with both primary and secondary resistance mutations. These viruses are resistant not only to the drug being given, but also to other protease inhibitors that the patient has never received. Monotherapy with indinavir, for example, can result in the development of virus that is resistant to indinavir and to other protease

inhibitors. This pattern also occurs with ritonavir monotherapy and could presumably be caused by monotherapy with any HIV-protease inhibitor.[53]

Integrase is one of three viral enzymes essential for HIV replication, catalysing the insertion of HIV DNA into the genome of the host cell. Integration is required for stable maintenance of the viral genome, as well as for efficient viral gene expression and replication. Because HIV-1 integrase represents a distinct therapeutic target, HIV-1 integrase inhibitors would be expected to maintain activity against HIV-1 resistant to the other classes of antiretroviral drugs. Raltegravir is an HIV-1 integrase strand transfer inhibitor that has been shown to be active against multidrug-resistant HIV-1. This inhibitor is additive or synergistic in vitro with currently available antiretroviral drugs. Strains of HIV resistant to Raltegravir remain susceptible to other agents. The drug has been shown to have potent antiretroviral effects, with a mean decrease from baseline in HIV-1 RNA concentrations of about 2 log10 copies/mL after 10 days of monotherapy.[54]

Therefore, there is great interest in searching for drugs with new target to be used in combination in order to overcome HIV resistance.

10.0 NCp7 as a new target

Due to the search for new antiviral agents that are able to overcome the critical problem of acquired resistance, one strategy could be that of developing inhibitors targeting viral proteins whose biological functions are ensured by domains that cannot be modified without a complete loss of activity. The HIV-1 nucleocapsid protein NCp7, could be such a new target. Indeed, NCp7 possesses a three-dimensional structure centered around two highly conserved zinc fingers, and nuclear magnetic resonance studies associated with site-directed mutagenesis have shown that any modification of this structure leads to a complete loss of HIV-1 infectivity.

Different classes of NCp7 inhibitors have been developed including: 1) structural peptido-mimetics of the essential biological determinants of NCp7, rationally developed by using the NCp7 structure; 2) chemicals proved to be able to destroy NCp7 folding by ejecting the zinc atom. These compounds display antiviral properties by disrupting different steps of the retroviral life cycle. Moreover, virions with inactivated NCp7 have been recently proposed for putative vaccines and immunological approaches.[37]

The NCp7 nucleocapsid protein of HIV-1 is a small 55-amino-acid protein generated by the viral protease-mediated cleavage of the Gag polyprotein precursor (Pr55gag), and is characterized by two highly conserved zinc fingers with a nonclassical Cys-Xaa2-Cys-Xaa4-His-Xaa4-Cys (CCHC)

motif flanked by basic amino acids on each side. The CCHC zinc-fingers consist of 14 aminoacids that chelate zinc through three cysteine thiolates and one histidine imidazolate. Each retroviral zinc finger coordinates one zinc ion with high affinity via CCHC-Zn^{2+} interactions, which act as the main driving force behind the folded structure of this small peptidic domain. Binding of an oligonucleotide to NCp7 modifies the architecture of the latter, notably at the linker and the N-terminal region. The linker becomes orthogonal to the ribose phosphate and locates the Zinc finger in preparedness to pinch the oligonucleotide. The interaction is mainly due to the Zinc finger tips so that a part of the Zinc finger structure remains accessible for additional interactions that are critical for viral replication. In addition, the spatial proximity of the two Zin fingers is reinforced on oligonucleotide binding. Nuclear Magnetic Resonance shows that the hydrophobic plateau at the surface of Zinc fingers represents the oligonucleotide –binding motif[55] (Figure 8).

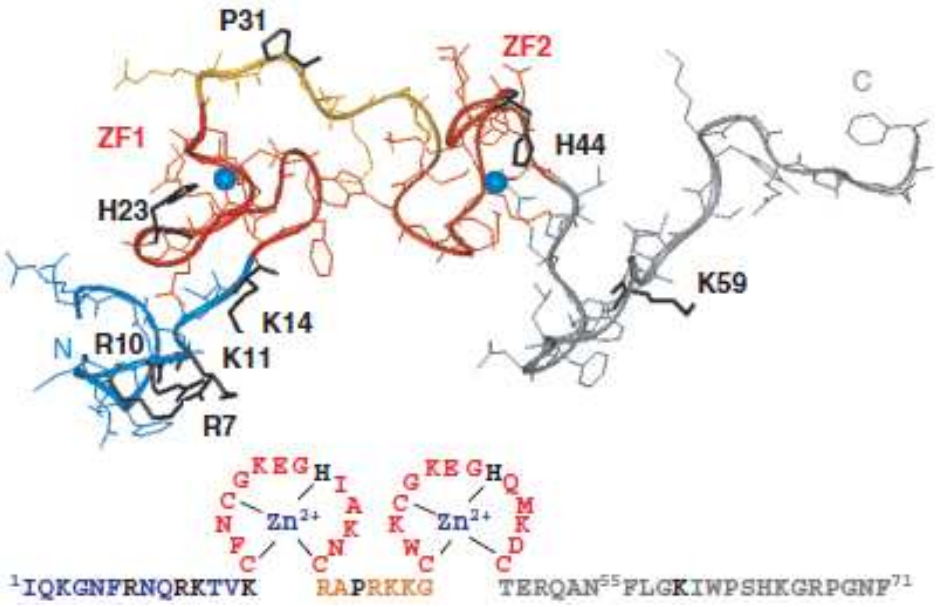


Figure 8. HIV nucleocapsid protein NCp7

The NCp7 protein is involved in several nucleic acid interacions during the early (from cell entry to proviral DNA integration) and late phases (viral protein synthesis and virion formation) of HIV-1 replication. The essential roles can be resumed as follows.

- Protection of the viral RNA from nucleases.[57]
- Promotion of the binding of the essential tRNA primer to the primer site.[57]
- Stimulation of reverse transcription.[57]
- Increase of the efficiency of template switching during reverse transcription. [58]
- Promotion of initiation and elongation of the nascent transcript.[58]
- Enhancement of integration of the viral DNA into the host chromosome.[59]
- Maturational cleavage of gag polyprotein.[58]

- Selection of viral RNA for dimerization.[57]
- Viral RNA packaging.[60]

Thus, the central role of the NCp7 protein and the absolute requirement for intact NCp7 protein and Zn fingers during HIV replication make this protein an attractive target[56] for drug discovery and development. A diverse set of electrophilic compounds that react with cysteine thiolates in retroviral nucleocapsid (NC) proteins and abolish virus infectivity has been identified. Although different in chemical composition, these compounds are all oxidizing agents that lead to the ejection of Zn ions from zinc finger viral proteins.[61]

11.0 NCp7 inhibitors

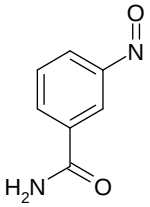
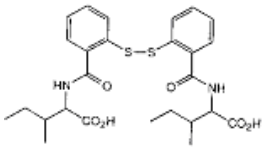
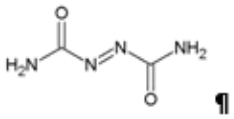
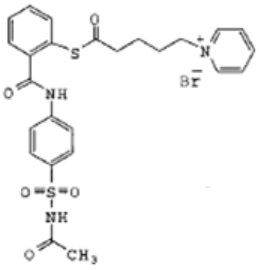
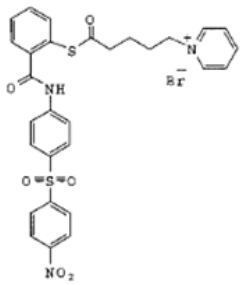
Several classes of compounds (reported in the following table 1) have been designed to inhibit NCp7. These compounds act primarily by covalently modifying zinc-coordinating cysteines, resulting in ejection of zinc and loss of protein function. Initial studies examined molecules based on 3-nitrosobenzamide (NOBA) which were highly active (EC_{50} = 1.5 µM) against NCp7,[52] but also reacted non-specifically with cellular zinc-binding proteins. Further studies led to the development of compounds with greater selectivity, such as the disulfide benzamide (DIBA) and azodicarbonamide (ADA) compounds. DIBA-4 compounds were highly active against NCp7 (EC_{50} of 1.2 µM) and non-cytotoxic up to 100 µM[63] in cell culture experiments. It has been proposed that the DIBA compounds function via an oxidative mechanism in which the sulfur atom from one of the zinc-coordinated cysteine residues slowly reacts with the disulfide moiety of the DIBA compound to form a mixed disulfide bond . A similar mechanism has been proposed for ADA compounds.[64] Mass spectrometry demonstrated that both Cys36 and Cys49 of ZD2 were covalently modified by the DIBA compounds.[65] Despite their selectivity, the DIBA compounds had the disadvantage that they contained an easily reduced disulfide bond that severely limited the half-life of the compound.[66] More recently, molecules based on a mercaptobenzamide thioester (for example the Pyridinio Alkanoyl Thioester (PATEs)) have been developed to inactivate HIV-1 by targeting NCp7. These compounds are more water-soluble than the DIBA compounds and eject coordinated zinc from NCp7. In addition, they have potent antiviral activity with low cytotoxicity in cell culture and animal models. These compounds preferentially interact with the second, C-terminal zinc-binding domain (ZD2) over the first, N-terminal zinc-binding domain (ZD1). It has been proposed that the thiol group of a zinc-coordinated cysteine ligand nucleophilically attacks the carbonyl carbon of the thioester compound resulting in covalent modification of the cysteine sulfur via an

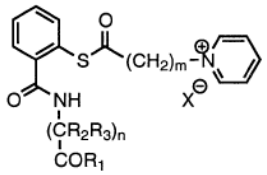
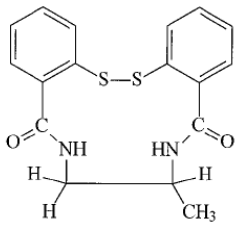
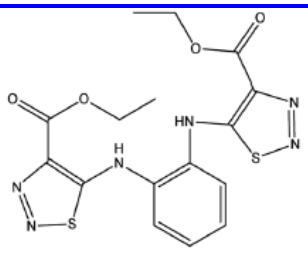
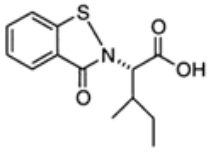
acyl transfer mechanism. This initial acylation weakens zinc coordination at the site of reaction. Subsequently, more acyl transfer reactions occur with other cysteine residues in the domain, which results in metal ejection, protein unfolding, and loss of function. Thus, the 2-mercaptobenzamide thioesters inactivate NCp7 by a mechanism different from previous inhibitors.[64]

Other studies have demonstrated that the 2,2'-dithiobisbenzamides (SRR-SB3) with an EC_{50} of 1.8 µM, cause extrusion of zinc from the zinc fingers of NCp7, resulting in a functionally ineffective form of NCp7. Moreover, they showed that this class of disulfides impairs the ability of HIV-1 virions to initiate reverse transcription through their action on the retroviral zinc finger, thereby blocking further rounds of replication.[67]

Another inhibitor is the NV038 (N,N'-bis(1,2,3-thiadiazol-5yl)benzene-1,2-diamine), that efficiently blocks the replication of a wide spectrum of HIV-1, HIV-2 and SIV strains. This compound interferes with a step of the viral replication cycle subsequent to the viral entry, but preceding or coinciding with the reverse transcription, and chelates Zn^{2+} from the NCp7 Zinc finger.[68]

A class of substituted 2'-benzisothiazolone represented by PD 161374 (2'-isothiazolone derivative of PD 159206) has been proved to possess an antiviral activity against HIV-1 similar to that of the above mentioned nucleocapsid inhibitor PD 159206 (DIBA-4). In T cell culture, PD 161374 showed an EC_{50} of 1.3 µM and no cytotoxicity up to 100 µM. It inhibited acute HIV infection and was effective when added during the early phases of HIV infection. PCR analysis of infected cells demonstrated that PD 161374 delayed the appearance of completed HIV–cDNA products including 2-LTR circles, thus suggesting that it exerts its antiviral effect at pre-integration steps in the early phase of the virus life cycle. PD 161374 was also evaluated in phase 1 clinical trials; however, due to toxicity, it was discontinued from further development.[63]

Chemical class	Lead compound structure	In vitro potency& cytotoxicity
3-nitrosobenzamide (NOBA)	 NSC 674195	EC50 = 1.56 µM CC50 = 50 µM [62]
Disulfide benzamide: 2,2'-dithiobisbenzamide (DIBA)	 DIBA-4 (PD 159206)	EC_{50} = 1.2 µM CC_{50} > 100 µM [63]
A-carbonyl azoic compounds: Azodicarbonamide (ADA)	 NSC 674447	EC_{50} = 43 µM CC_{50} = 184 µM [69]
Pyridinioalkanoyl thiolester (PATE)	 *N*-[2-(5 pyridiniovaleroylthio) benzoyl]sulfacetamide bromide  *N*-[2-(5-pyridiniovaleroylthio)-benzoyl]-4-(4-nitrophenylsulfonyl)aniline bromide	EC50= 6.2 µM CC50> 316 µM [70] EC50= 5.5 µM CC50> 316 µM [70]

		EC50= 0.8-9.8 µM CC50 = 43 - > 100 µM [71]
Macrocyclic 2,2'-dithiobisbenzamide	 SRR-SB3	EC50= 1.8 µg/ml CC50 = 15.9 µg/ml [67]
N,*N*'-bis(1,2,3-thiadiazol-5-yl) benzene-1,2-diamine	 **NV038**	EC50=17±3 µM CC50= 297.5 µM [68]
2'-benzisothiazolone (PD161374) derivatives of DIBA		EC_{50}= 1.3 µM CC_{50}>100 µM [63]

12.0 Materials and Methods

Compounds. Compounds were solubilised in DMSO at 100 mM and then diluted in culture medium.

Cells and Viruses. MT-4, C8166, H9/III_B and CEM cells as well as laboratory-adapted HIV-1 strains were obtained from NIH AIDS Research &Reference Reagent Program, USA. Cell cultures were grown in RPMI 1640 medium, supplemented with 10% foetal calf serum (FCS), 100 IU/mL penicillin G and 100 µg/mL streptomycin, and incubated at 37 °C in a 5% CO_2 atmosphere. Cell cultures were checked periodically for the absence of mycoplasma contamination with MycoTect Kit (Gibco).

HIV-1 $_{IIIB}$ and HIV-2 CBL-20 strains were obtained from supernatants of persistently infected H9/III_B and CEM cells, respectively. HIV-1 and HIV-2 stock solutions had titres of 4.5 x 10^7 and 1.4 x 10^6 50% cell culture infectious doses ($CCID_{50}$)/mL, respectively. The Y181C mutant (NIH N119) derives from an AZT-sensitive clinical isolate passaged initially in CEM and then in MT-4 cells, in the presence of Nevirapine (up to 10 μM). The K103N + Y181C mutant (NIH A17) derives from an III_B strain passaged in H9 cells in the presence of BI-RG 587 (up to 1 μM). The K103R + V179D + P225H mutant (EFV^R) derives from an III_B strain passaged in MT-4 cells in the presence of Efavirenz (up to 2 μM). N119, A17 and EFV^R stock solutions had titres of 1.2 x 10^8, 2.1 x 10^7, and 4.0 x 10^7 $CCID_{50}$/mL, respectively. Mutants carrying NRTI mutations, such as the AZT^R strain (67N, 70R, 215F, 219Q) and the MDR strain (74V, 41L, 106A, 215Y) or PRI mutations, such as SAQ^R (L10F, G48V, L90M), were also tested.

Anti-HIV Assays. The activity of test compounds against the replication of HIV-1 wt and mutant strains (N119, A17, EFV^R, AZTR, MDR) and HIV-2 wt in acutely infected cells was based on inhibition of virus-induced cytopathogenicity in MT-4 and C8166 cells, respectively. Briefly, an amount of 50 μL of culture medium containing 1 x 10^4 cells were added to each well of flat-bottom microtitre trays containing 50 μL of culture medium without or with different serial concentrations of test compounds. Then, 20 μL of an HIV suspension (containing the appropriate amount of $CCID_{50}$ needed to cause complete cytopathogenicity at day 4) were added. After incubation at 37 °C, cell viability was determined by the 3-(4,5-dimethylthiazol-1-yl)-2,5-diphenyltetrazolium bromide (MTT) method[72]. The cytotoxicity of test compounds was evaluated in parallel with their antiviral activity through the viability of mock-infected, treated cells, as monitored by the MTT method, and described elsewhere.[73]

Reverse Transcriptase Assays. Assays with recombinant HIV-1 reverse transcriptase were performed as previously described.[74, 75]

Integrase Assays. Expression and purification of HIV-1 recombinant integrase (rIN), as well as rIN assays have were done as previously described.[75]

p24 Determination. Quantitation of the p24 present in supernatants of HIV-1 infected MT-4 cells was made using the Abbott HIVAG-1 monoclonal solid phase enzyme immunoassay, according to the manufacturer's protocol.

H9/C8166 Cocultures. Chronically infected H9 cells were washed and cocultured with uninfected C8166 cells (ratio 1:500) in the absence or in the presence of test inhibitors. Following 36 hrs incubation at 37 °C, cocultures were monitored by optical microscopy, syncytia were counted and those found in drug treated cocultures were reported as a percentage of those counted in untreated cocultures.[76]

Time of Addition Assays. 2 x 10^4 MT-4 cells were infected for 1 h at 20°C with HIV-1 at an high multiplicity of infection (m.o.i. > 5 cell culture infectious dose fifty ($CCID_{50}$)/cell). Test compounds were added in duplicate at the beginning of the 1 hr infection period (and then removed together with the inoculum by extensive washing), or immediately after the end of infection, 1, 2, 4, 6, 8, 12 or 24 hrs later. The amount of p24 and infectious virus present in supernatants were measured 42 hrs p.i..

Selection of Drug-Resistant Mutants. Drug-resistant variants were selected by serial passages of HIV-1_{IIIB} in the presence of stepwise doubling drug concentrations, starting from a cell culture infected with an m.o.i. of 0.01, and treated with a drug concentration equal to the EC_{50}. Usually, the amount of virus obtained after each passage was sufficient to determine infection of the next cell culture which, after infection and washing, was incubated with a double amount of the selecting drug. In the case of compound 6, the drug-resistant virus population was selected up to a drug concentration 8-fold greater than the EC_{50}. Resistant virus preparations were subjected to RNA extraction, RT-PCR and genome sequencing to identify the mutation patterns responsible for resistance.

Molecular Analysis of Resistant Viruses. Viral RNAs from wt and drug-resistant mutants were obtained using the QIAamp viral RNA minikit (QIAGEN), starting from 140 μL of cell-free viral suspensions containing about 5 x 10^4 PFU/mL, in order to determine the nucleotide sequence of the *pol*, *gag* and *env* regions of HIV-1 genome. Reverse transcriptions were carried out using the Superscript II enzyme (INVITROGEN) and cDNAs were amplified by PCR using Platinum *Pfx* polymerase (INVITROGEN) following the manufacturer's protocol. Details of the RT-PCR conditions are reported in the Supporting Information section.

PCR fragments were purified using the QIAquick PCR Purification kit (QIAGEN) and analyzed using the cycle-sequencing method (CIBIACI service of University of Firenze). Both DNA strands

were sequenced with specific primers. The comparative analysis of the chromatograms allowed us to deduce the mutation patterns responsible for resistance to compound 6 (HIV-1[6]).

Primers and parameters used in the RT-PCR reactions. The primer used in the RT reaction were: i) OUTR 5'-CATTGCTCTCCAATTACTGTGATATTTCTCATG-3', that binds about 400bp downstream the RT gene, and ii) ENVR6 5'-CTGCTGTGTTGCTACTTGTGATTG-3', that binds about 160bp downstream the *env* gene. cDNAs were amplified by PCR using Platinum *Pfx* DNA Polymerase (INVITROGEN), following the manufacturer's protocol. The regions containing the *protease* and *RT* genes were amplified by PCR reactions carried out with the primers:
INF (5'-TGAAAGATTGTACTGAGAGACAGG-3') and
INR (5'-TCTATTCCATCTAAAAATAGTATT TTCCTGATTCC-3'), respectively.
PCR consisted of an initial denaturation step of 3 min; 30 cycles of denaturation at 94 °C for 30s, annealing at 52 °C for 30s and extension at 68 °C for 2.5 min; final extension at 68 °C for 5 min. The *gag* region was amplified by two separated PCR reactions, carried out with the primers:
GF1 (5'-GCGACTGGTGAGTACGCCAAAAAT-3') and
GR2 (5'-CAGCCAAAACTCTTGCCTTATGGC-3') and with the primers
GF2 (5'-TCAGCCCAGAAGT GATACCCATGT-3') and
GR1 (5'-GGTTTCCATCTTCCTGGCAAACTC-3'), respectively,
under the conditions described above except that the annealing temperature was 51.5 °C and the extension time was 1.5 min. The *env* region was amplified by two different PCR reactions, carried out with the primers EF1:
(5'-CTCAAGGCAGTCAGACTCATCAAG-3') and
ER2 (5'-TTGGTGGGTGCTACTCCTAATGGT-3') and with the primers
EF2 (5'-GAGGACCAGGGAGAGCATTTGTTA-3') and
ER1 (5'-AGCAGGCCATCCAATCACACTA CT-3') respectively,
under PCR amplification conditions similar to the first one described above, except that the annealing temperature was 54 °C and the extension time was 1.5 min.

Purification of PCR products. The cDNA products were purified, to remove all PCR components, using a QIAquick PCR Purification Kit (QIAGEN) and following the manufacturer's recommendations. 5 volumes of PB buffer were added to each PCR sample. The sample was applied to the QIAquick column and centrifuged for 1 min at 13000 rpm at rt. Washing was done with 750 µl of PE buffer, centrifuging for 1 min at 13000 rpm at rt, discarding the flow-through and centrifuging for 1 additional minute to completely remove the residual ethanol from the PE buffer.

The cDNAs were finally eluted from the column with 50 µl of DNase-free water, centrifuged at 13000 rpm for 1 min at room temperature and stored at –20 °C.
DNA quantitative determination The concentration of purified cDNAs was checked by the spectrophotometer method (Eppendorf Biophotometer). The nitrogenous bases of nucleotides have in fact the absorption maximum at about 260 nm. Using a 1-cm light path, the extinction coefficient for nucleotides at this wavelength is 20 and based on this extinction coefficient, the absorbance at 260 nm in a 1-cm cuvette of a 50 µg/ml solution of double stranded DNA results to be 1 (50 µg/ml DNA : $1A_{260nm}$ = x µg/ml DNA : A_{260nm} of the sample).

DNA sequencing. Purified PCR fragments were sent to C.I.B.I.A.C.I. of Firenze University for sequencing. Samples were prepared adding in a 0.2 ml tube, 10 ng for 100 pb of purified DNA fragment and 5 pmol of specific primer; then samples were incubated at 65°C for 30 minutes to allow the water evaporation and to freeze-dry the mix cDNA-primer. Sequencing was performed on both strands of DNA fragments using primers designed with a distance of about 500 nt, since that a good lecture of chromatogram is possible for about 600-700 nt.
Analysis of all received chromatograms allowed us to obtain the entire DNA sequence of F, G, SH and L genes; the comparative analysis between wild-type and drug-resistance strains allowed us to identify the mutations selected during the resistance acquisition.

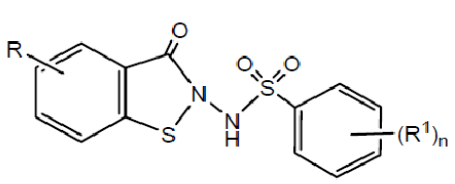
Virucidal Activity Assays. Cell-free, high titre HIV-1 and HIV-2 stock solutions were exposed to test compounds for 2 hrs at 0 or 37 °C. At the end of incubation, the residual infectivity was determined by the Reed and Muench titration[77], as described below.

HIV Titration. Titration was performed in C8166 cells by the standard limiting dilution method (dilution 1:2, four replica wells/dilution) in 96-well plates. The infectious virus titre was determined by light microscope scoring of syncytia (multinucleated giant cell) after 4 days of incubation. Virus titers were expressed as 50% cell culture infectious dose per mL by the Reed and Muench method.[77]

13.0 RESULTS

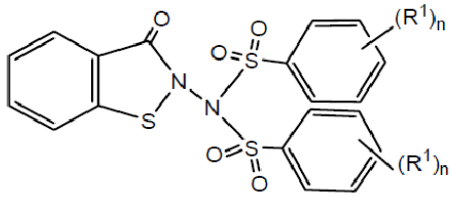
Premises

The present study deals with the attempt of exploring the potential of a new class of small molecule HIV inhibitors, the 1,2-benzisothiazol-3(2H)-one benzenesulfonamides, which emerged in the framework of our previous research on biologically active benzisothiazole derivatives.[13-17] The synthetic modulation of the basic scaffold, aimed at elucidating SAR analysis, led to structures **6-24** (Figure 9), which are endowed with antiretroviral activity *in vitro* at micromolar level. Their potency is in the range reported by other Authors for benzisothiazolones and for NC inhibitors belonging to other classes (for review see [6] and references therein). However, in addition to being capable to prevent, in cell-based assays, the replication of HIV type-1 (HIV-1), type-2 (HIV-2) and HIV-1 strains resistant to the major drug classes currently used in clinic, they show concentration-dependent inactivation of the infectivity of cell-free HIV-1 and HIV-2.



6-19

	n	R	R^1
6	0	H	-
7	1	H	4-CH_3
8	1	H	4-NO_2
9	1	H	4-OCH_3
10	1	H	2-F
11	1	H	4-F
12	1	H	2-Cl
13	1	H	3-Cl
14	1	H	4-Cl
15	2	H	3,4-Cl_2
16	2	H	3,5-Cl_2
17	1	5-CH_3	2-Cl
18	1	5-Cl	2-Cl
19	1	6-Cl	2-Cl



20-24

	n	R^1
20	0	-
21	1	4-CH_3
22	1	4-NO_2
23	1	4-F
24	1	4-Cl

Figure 9. Chemical structures of 1,2-benzisothiazol-3(2H)-one benzenesulfonamides ***6-24****.*

Chemistry

The key intermediates 2,2'-dithiobisarylcarboxylic acid 1a-1d, required for the synthesis of compounds **6-24**, were prepared, when not commercially available, according to the known procedure outlined in Figure 10A. The suitable substituted 2-aminobenzoic acid was diazotized and then converted, by a Sandmeyer reaction using ethylxantogenate, into the ester intermediate. Next, upon alkaline hydrolysis and subsequent acidification, the 2-mercaptoarylcarboxylic acid was obtained. Oxidation by iodine afforded the key intermediate **1a-1d**.[78]

To prepare the target compounds **6-24**, the 2,2'-dithiobisarylcarboxylic acid 1 was treated with thionyl chloride affording the dimeric acid chloride **2**, which was then converted into the chlorocarbonylphenylsulfenylchloride **3** by treatment with dry chlorine. A subgroup (unsubstituted and substituted *N*-(3-oxo-1,2-benzisothiazol-2(*3H*)-yl)benzenesulfonamides **6-19**) of the target compounds could be obtained from two alternative routes **A** and **B** (Figure 10B). **A**: Upon cyclization of **3** with *N*-protected hydrazine the intermediate **4** resulted, then the *N*-Boc group was removed by mild acidic hydrolysis with trichloroacetic acid (TCA) to yield the 2-amino-1,2-benzisothiazol-3(*2H*)-one **5**, according to the method previously described by Vicini *et al*.[79]

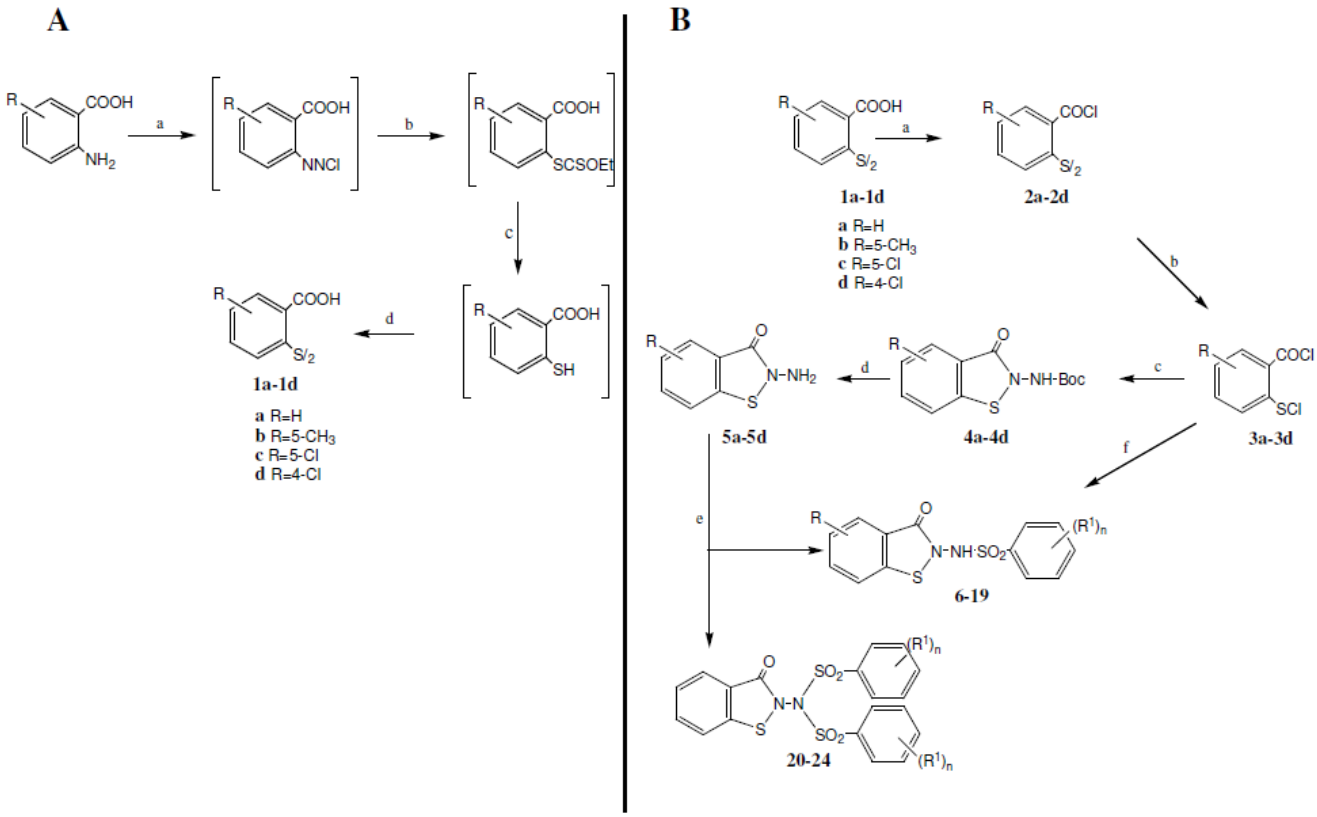


Figure 10. Chemical synthesis. A) Synthesis of the key intermediates 1a-1d. Reagents and conditions: a) $NaNO_2$, HCl, 0 to 5 °C; b) KS-CSOEt , 70-80 °C; c) aq NaOH 10%, reflux, 4 h; conc. HCl; d) I2, ethanol, 50 °C. B) Synthesis of the target compounds 6-24. Reagents and conditions: a) $SOCl_2$, reflux, 3h; b) Cl_2, 3h, RT; Procedure A; c) NH_2-NH-Boc;

$(C_2H_5)_2O$/pyridine, 60 min, 10 °C; d) TCA/H_2O, 150 min, RT; e) (R1)nphenyl-SO_2-Cl, pyridine, 60-120 min, 10 to –5 °C; Procedure B; f) (R1)nphenyl-SO_2-NH-NH_2, CCl_4/pyridine, 120 min, 0 °C.

Reaction with the appropriate benzenesulfonylchloride, in pyridine, in a cooling bath, afforded a mixture of *N*-(3-oxo-1,2-benzisothiazol-2(*3H*)-yl)benzenesulfonamides **6**-**19** and of the respective *N*-(3-oxo-1,2-benzisothiazol-2(*3H*)-yl)-*N*-(phenylsulfonyl)benzenesulfon- amides (yield ratio 20-50%: 50-80%). Indeed, once the sulfonamide **6**-**19** is formed, it readily undergoes, by the action of the electrophilic sulfonyl chloride, a subsequent sulfonylation yielding the bisbenzenesulfonyl derivative. The final *N*-(3-oxo-1,2-benzisothiazol-2(*3H*)-yl)benzenesulfonamide and the respective *N*-(3-oxo-1,2-benzisothiazol-2(*3H*)-yl)-*N*-(phenylsulfonyl)benzenesulfonamide could any way be simply separated because of the acidic character of the former. **B**: Direct cyclization of the chlorocarbonylsulfenyl chloride 3 with the suitable benzenesulfonohydrazide afforded the desired product **6**-**19**. Surprisingly, the latter procedure did not increase the yield of target compounds; therefore, the procedure A was preferred, being the substituted benzenesulfonyl chlorides more easily available.

The *N*-(3-oxo-1,2-benzisothiazol-2(*3H*)-yl)-*N*-(phenylsulfonyl)benzenesulfonamides **20**-**24**, selected for the present study, could be obtained in high purity after re-crystallization of the alkali insoluble residue formed as the final product of procedure A (Figure 10B).

Biology

Data on cytotoxicity (CC_{50}), potency (EC_{50}) and spectrum of antiretroviral activity of 1,2-benzisothiazol-3(*2H*)-one benzenesulfonamides (derivatives **6**-**24**) are reported in Table 1. The HIV-1 strains tested include: wild type III_B and mutants resistant to NNRTIs, NRTIs and PRIs, as detailed in Material and Methods. The activity against HIV-2 (CBL-20 strain) in C8166 cells is also reported. In this study, benzenesulfonamides were evaluated in comparison with the following reference drugs: efavirenz (EFV), azidothymidine (AZT) and saquinavir (SQV). Most of title derivatives resulted effective, in the micromolar concentration range, against HIV-1 wt and variants carrying clinically relevant mutations which confer resistance to the three major classes of anti-retrovirals. Moreover, unlike EFV, compounds **6**, **7**, **17** and **19** also inhibited HIV-2 (Table 1), thus showing broad-spectrum antiretroviral activity.

Selected *N*-(3-oxo-1,2-benzisothiazol-2(*3H*)-yl)benzenesulfonamides, i.e. compounds **6**, **7**, **17** and **19**, were further tested, as described in our previous work, in enzyme assays aimed at evaluating their capability to inhibit recombinant HIV-1 RT[74] and IN.[75] Interestingly, unlike reference drugs [EFV (IC_{50} on rRT = 0.06 µM) and the diketoacid inhibitor of rIN, L-731,988 (IC_{50}s on 3' processing and strand transfer = 2.5 and 0.35 µM, respectively)], none of the benzenesulfonamides

resulted active at concentrations of 30 µM (results not shown), thus making it unlikely that RT or IN might be their target enzymes.

Taken together, the results obtained in cell-based and enzyme assays suggest that benzenesulfonamides have a mode of action different from that of drugs belonging to the major antiretroviral classes.

Table 1. Cytotoxicity and antiretroviral activity of compounds **6**-**24** and reference drugs.

Compds	[a]CC_{50} [µM]	[b]EC_{50} [µM]							
	MT-4	[c]HIV-1	[d]N119	[d]A17	[d]EFV^R	[e]AZT^R	[e]MDR	[f]SAQ^R	[g]HIV-2
6	86	8	7	8	7	6	4	8	7
7	35	10	5	8	8	8	6	9	4
8	>100	15	7	11	9	7	6	8	[h]ND
9	56	8	6	8	6	5	6	7	ND
10	>100	7	6	7	5	6	5	7	ND
11	50	5	5	5	2	5	6	4	ND
12	73	13	7	11	9	9	7	9	ND
13	33	11	6	8	3	7	7	8	ND
14	38	5	7	3	7	6	6	4	ND
15	66	15	7	9	8	6	11	8	ND
16	86	16	7	11	10	8	13	12	ND
17	73	2	5	9	2	4	3	6	4
18	>100	18	14	20	6	12	16	15	ND
19	54	11	14	≥33	2	14	11	21	12
20	>100	24	23	25	22	20	19	25	ND
21	>100	76	44	58	70	70	63	63	ND
22	>100	21	20	20	21	24	25	23	ND
23	34	26	23	24	25	22	25	22	ND
24	>100	71	29	56	65	71	64	63	ND
EFV	30	0.002	0.008	0.3	3	0.002	0.004	0.007	>20
AZT	≥20	0.01	0.02	0.01	0.01	0.2	0.2	0.01	0.008
SAQ	>20	0.01	0.02	0.01	0.01	0.01	0.02	1.0	ND

[a]Compound concentration [µM] required to reduce the viability of mock-infected MT-4 cells by 50%, as determined by the MTT method.

[b]Compound concentration [µM] required to achieve 50% protection of MT-4 and C8166 cells from the HIV-1- and HIV-2-induced cytopathogenicity, respectively, as determined by the MTT method. Data represent mean values for three independent determinations. Variation among duplicate samples was less than 15%.

[c]HIV-1: IIIB strain.

[d]NNRTI resistant mutants: N119 (Y181C); A17 (K103N+Y181C); EFV^R (K103R+V179D+P225H).

[e]NRTI resistant mutants: AZT^R (67N, 70R, 215F, 219Q); MDR (74V, 41L, 106A, 215Y).

[f]PRI resistant mutant: SAQ^R (L10F, G48V, L90M).

[g]HIV-2: CBL-20 strain.

[h]ND: not determined.

The potential interference of compound **6** with processes similar to those leading to HIV entry into susceptible cells was evaluated in cocultures of HIV-1 chronically infected H9 cells with uninfected

C8166 cells.[22] In this assay, syncytia are generated as a result of the interaction of *env*-encoded glycoproteins (present on the outer membrane of chronically infected H9 cells) with the CD4 of cocultured C8166 cells. Contrary to dextran sulphate, 0.5 μM, that prevented syncytia formation by 98%, **6**, 30 μM, failed to interfere with the very early events (adsorption/attachment/fusion) in the HIV multiplication cycle triggered by the gp120-CD4 interaction[80, 81] (results not shown).

In the attempt to identify the step(s) of the HIV-1 infectious cycle target of compound **6**, a time of addition (see Materials & Methods) study was carried out in MT-4 cells under a single round of viral replication, in comparison with drugs representative of the NNRTI, NRTI and PRI antiretroviral classes. The amount of p24 (Figure 11) and infectious virus (Table 2) present in supernatants were measured 42 hrs p.i..

Table 2. HIV-1 titres in supernatants of cultures treated during infection or at various times p.i. with compound **6** and EFV.

Time of drug addition	Infectious virus titre ($CCID_{50}$/mL) at 42 hrs p.i.	
	Compound 6 (15 μM)	EFV (0.05 μM)
drug present only during the infection period (-1 – 0)	5.4×10^6	8.2×10^6
0 hrs p.i.	1.0×10^4	6.3×10^3
4 hrs p.i.	2.0×10^4	6.8×10^3
8 hrs p.i.	8.5×10^3	6.8×10^5
12 hrs p.i.	2.1×10^4	7.1×10^6
24 hrs p.i.	1.8×10^5	1.1×10^7
Untreated control	8.5×10^6	

When present only during the 1 hr infection period, none of the compounds tested inhibited the HIV-1 multiplication, as revealed by p24 production (results not shown) or infectious virus yield. In particular, both EFV and **6** allowed an infectious virus yield similar to that of untreated, infected controls (Table 2), suggesting that, in addition to not affecting HIV-1 entry, compound **6**, 15 μM, did not accumulate and persist into the cells. In order to significantly inhibit HIV-1 multiplication, and due to the need to be activated by cellular kinases into its triphosphate form, AZT had to be added within 4 hrs p.i. as expected (Figure 11).

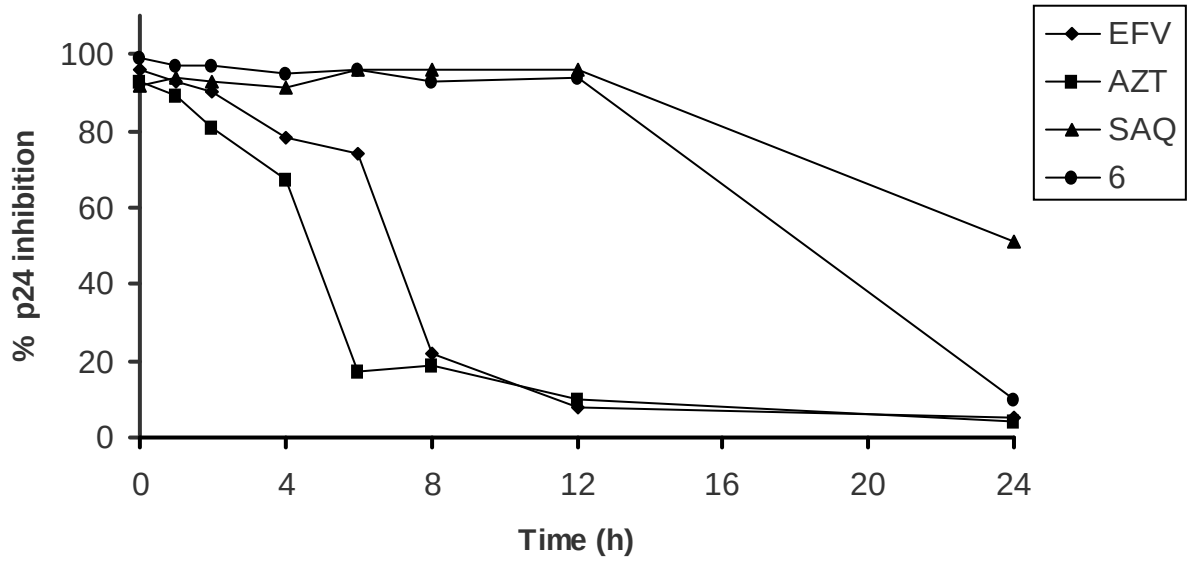


Figure 11. Time of addition studies comparing the effects, on p24 production at 42 hrs p.i., of compound 6, azidothymidine (AZT), efavirenz (EFV) and saquinavir (SAQ).

On the contrary, being capable of targeting RT without prior metabolization, EFV was still inhibitory even if added within **6** hrs p.i. (Figure 11 and Table 2), suggesting that, under the experimental conditions used, reverse transcription was essentially completed by **6** hrs p.i. Interestingly, like SAQ, **6** resulted effective in preventing both p24 production (Figure 11) and virus yield (results not shown), no matter whether it was added to the cultures immediately at the end of the infection period or any time up to 12 hrs later. On the other hand, when added 24 hrs p.i., **6** did not prevent p24 production (Figure 11), but was significantly effective in reducing the infectious HIV-1 yield (Table 2), whereas SAQ was effective in inhibiting both p24 production (although by 50%, Figure 11) and infectious virus yield (results not shown). These data suggested that **6** targets events occurring both early and late in the HIV infection cycle.

Mutants resistant to **6** were selected by serial passages of HIV-1 in the presence of stepwise doubling drug concentrations, starting from cell cultures infected with an m.o.i. of 0.01 and, for the first two passages, incubated with a drug concentration equal to the EC_{50}. Thereafter, the amount of virus obtained after each passage was sufficient to carry on the infection of the next cell cultures which, after infection and washing of the unadsorbed inoculum, were incubated with stepwise doubling amounts of the selecting drug. At passage n° 3 the drug concentration had to be kept unaltered in order to get enough virus to progress with the next selection passage. In the following passages, 6-resistant mutants (HIV-1^{6}) were grown up to a drug concentration 8-fold higher than the EC_{50}. The selected virus population ($5x10^4$ $CCID_{50}$/mL) was then subjected to RNA extraction, RT-PCR and sequencing of *pol*, *env* and *gag* genes in order to identify the mutation pattern responsible for drug resistance. Comparative genomic analysis of parental HIV-1 wt, subcultured for the same

time period without drug, and of the HIV-1^6 mutant showed that the latter contained no mutations in the RT and PR genes. The same was also true for the *env* gene of the HIV-1^6 mutant strain, suggesting that none of the proteins encoded by *pol* and *env* was the target of compound **6**. On the contrary, two mutations, F6C and R32K, were selected in NC domain of the *gag* gene of the mutant virus, (Figure 12) suggesting that NC proteins, which favour reverse transcription, integration, genome RNA packaging and virion assembly, are the likely target of **6**.

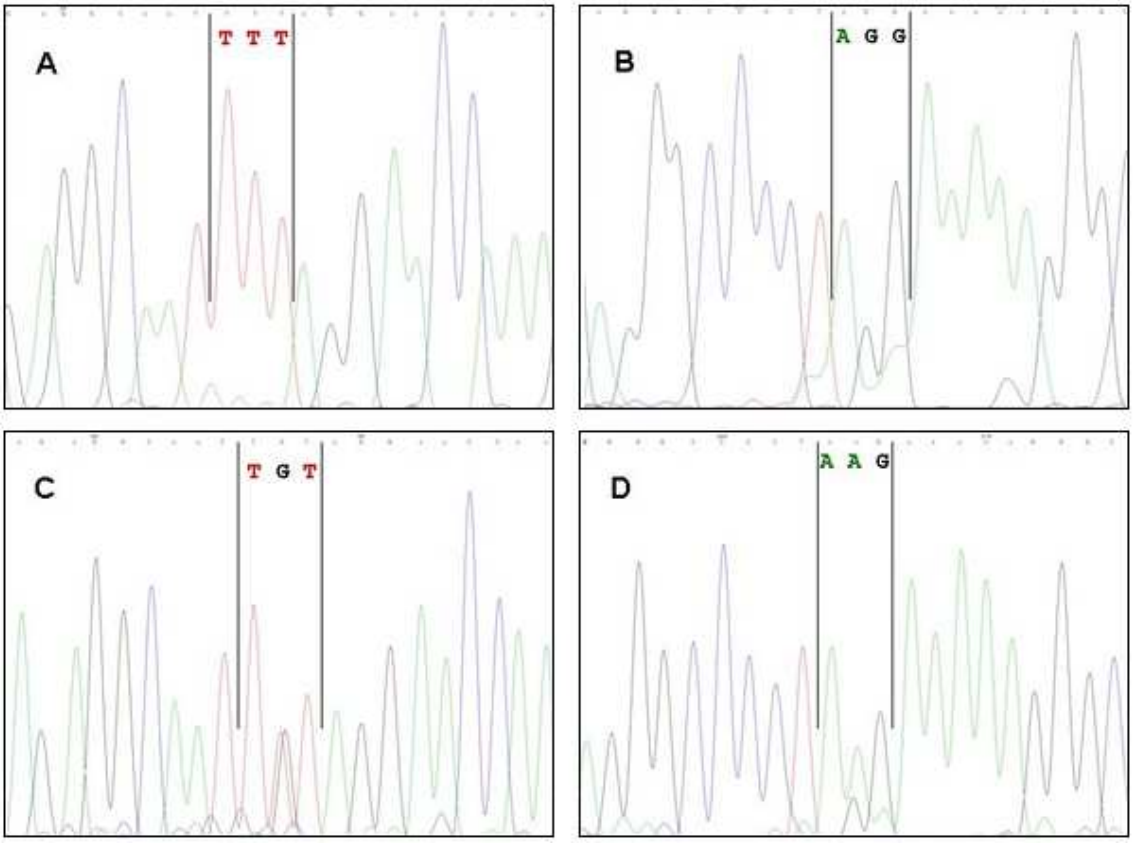


Figure 12. Chromatograms of NCp7 gene region containing nucleotides codifying for amino acids Phe-6 and Lys-32. HIVwt chromatograms (A, B) show respectively T-T-T and A-G-G, while HIV6 chromatograms (C, D) show respectively T-G-T and A-A-G.

NC inhibitors are reported[38] to directly inactivate the infectivity of cell-free HIV-1. Thus, in order to investigate the potential virucidal activity of our candidate compound, **6** was incubated for 2 hrs at 37 °C with an high titre HIV-1 stock solution, whose residual infectivity was then determined by the Reed and Muench end point titration method.[77] Amphothericin B, a cholesterol-depleting compound capable of inhibiting HIV-1 infectivity, was used as a reference drug because of its direct virucidal properties.[82] When used at concentrations about 10- and 100-fold higher than the

respective EC_{50}s, both **6** and amphothericin B turned out to be capable of inactivating the HIV-1 infectivity by more than 3 log_{10} (Table 3).

Table 3. Comparative virucidal activity of **6** and AMPH-B against HIV-1 and HIV-2.

	Incubation of HIV-1 stock solution for 2 hrs at:		Incubation of HIV-2 stock solution for 2 hrs at:	
	37 °C	0 °C	37 °C	0 °C
	Infectious virus titre ($CCID_{50}$/mL)			
6 (100 μM)	4.5×10^3	1.0×10^7	9.3×10^2	1.3×10^5
[a]AMPH-B (100 μM)	$\leq 10^2$	4.5×10^5	2.8×10^3	1.0×10^4
[b,c]Untreated controls	8.9×10^6	8.5×10^6	2.4×10^5	2.0×10^5

[a]EC_{50} of AMPH-B in cell based assays was 1 μM.
[b]HIV-1 stock solution thawed from -80 °C had a titre of 1.3×10^7 $CCID_{50}$/mL.
[c]HIV-2 stock solution thawed from -80 °C had a titre of 2.8×10^5 $CCID_{50}$/mL.

Interestingly, when the incubation was carried out at 0 °C, compound **6** failed to affect the HIV-1 infectivity, while amphothericin B was still able to reduce it by 95%. When the same type of experiment was carried out with HIV-2, both **6** and amphothericin B inactivated virion infectivity by approximately 2 log_{10} (Table 3). Again, if the incubation was carried out at 0 °C, compound **6** failed to significantly affect the HIV-2 infectivity, whereas amphothericin B reduced it by more than 1 log_{10} (Table 3). Contrary to amphothericin B, the binding of which alters the properties of the viral membrane, the temperature-dependent inactivation of the viral infectivity by **6** suggests that the benzisothiazole could have a mode of action different from disruption of virion morphology[82]. Infectivity inactivation of both HIV-1 and HIV-2 was then determined by exposing cell-free stock solutions to serial dilutions of **6**. As shown in Table 4, concentrations of 100 and 50 μM strongly inactivated the infectivity of both HIV types in a concentration–dependent manner; viceversa, lower concentrations (25 and 12.5 μM) had only marginal or no effects on the HIV infectivity. These results suggest that the inhibition of HIV-1 replication observed in cell-based assays (at 8 μM) is not due to the infectivity inactivation of virions.

Table 4. Concentration-dependent inactivation of cell-free, infectious HIV-1 and HIV-2.

Compound **6** [μM]	Incubation of stock solutions for 2 hrs at 37 °C	
	HIV-1	HIV-2
	Infectious virus titre ($CCID_{50}$/mL)	
100	3.7×10^3	9.3×10^2
50	4.3×10^4	7.9×10^4
25	1.0×10^5	1.2×10^5
12.5	9.0×10^5	2.8×10^5
[b]Untreated controls	2.7×10^6	2.8×10^5

14.0 DISCUSSION

Novel synthetic 1,2-benzisothiazol-3(*2H*)-one benzenesulfonamides with a wide spectrum of antiretroviral activity against HIV-1, HIV-2 and HIV-1 variants carrying clinically relevant mutations conferring resistance to NNRTIs, NRTIs and PRIs are described. They neither interfere with the gp120 - CD4 interaction leading to HIV entry and syncytium formation, nor inhibit RT or IN in enzyme assays. Nevertheless, they affect events occurring both early and late in the HIV multiplication cycle and inactivate the infectivity of cell-free HIV virions in a concentration–dependent manner. Two mutations, F6C and R32K, were detected in the *gag* region coding for NC proteins of mutants selected for resistance to 6. Therefore, since mutations evolve to counteract binding of the drug to its target, it is reasonable to suggest that, in wt viruses, NC proteins are the target of compound 6. The fact that NC proteins are involved in both early (reverse transcription[83,84] and integration[85]) and late (protease processing[86], packaging of viral genomic RNA[87]) stages of the HIV-1 replication cycle, in addition to being structural components of mature virions, is consistent with both inhibition of HIV multiplication in infected cells and direct inactivation of virion infectivity.

NC proteins of retroviruses are synthesized as functional domains of the Gag precursor. In HIV-1, mature NC is generated, late in the assembly before virus budding takes place, by a cascade of PR-mediated cleavages of $Pr55^{Gag}$. End-products are matrix ($p17^{MA}$), capsid ($p24^{CA}$) and nucleocapsid (NCp7) proteins, p6 and smaller peptides.[88]

NC proteins, no matter whether as domain of $Pr55^{Gag}$, or intermediate cleavage products ($p15^{NC}$ and $p9^{NC}$), or mature NCp7, are characterized by two zinc finger sequences $C\text{-}X_2\text{-}C\text{-}X_4\text{-}H\text{-}X_4\text{-}C$ (CCHC), which are highly conserved among retroviruses. The zinc finger sequences are linked by the central spacer RAPRKKG and flanked by the NH_2-terminal (MQRGNFRNQRKIVK) and COOH-terminal (TERQAN) regions, which are highly conserved within a particular retrovirus species. As a matter of fact, HIV-1 NC proteins function as nucleic acid chaperons; in fact, they destabilize nucleic acid helices (through zinc fingers) and elicit nucleic acid aggregation (through basic residues). NC proteins are absolutely required for viral replication, and genetic analyses have proved that many, even minor alterations can affect virus assembly by disrupting genomic RNA (gRNA) recognition and packaging, or formation of infectious particles[88], consistently with the results described in this paper.

Evidence for binding of isothiazolones to cysteine (Cys) of the NC Zn fingers domains has been claimed,[89] as well as evidence for the lability of their N-S bond subsequent to a nucleophilic attack by the sulphur atom of Cys at the sulphur of the isothiazolone moiety.[90] When binding to Cys occurs at the NC Zn-finger domain, zinc extrusion (and concurrent denaturation of the NC protein) is known to occur with several thio-compounds bearing proper electrophilic groups.[91,92,65] Therefore, it is reasonable to suggest that, because of their structure, also the 2-benzisothiazol-3(*2H*)-one benzenesulfonamides reported herein may affect NC functions by binding through the sulphur of the isothiazolone moiety at the Cys of the NC Zn fingers domains.

Numerous studies have described the importance of several NCp7 residues, i.e.: the zinc ligands (C15, C18, H23, C28, C36, C39, H44, C49), those of the finger domains (F16, W37),[73,93] those involved in the interaction with nucleic acids (V13, F16, A25, W37, M46)[94] and those at positions 37, 38 and 45 of the C-terminal zinc binding domain. In particular, mutations involving the zinc ligands and the aromatic residues of the finger domains have been proved to prevent provirus formation or lead to loss of infectivity due to defects in RNA protection and encapsidation.

Based on the above, NC proteins have been indicated as an ideal target for antiretroviral compounds characterized by high barrier to the development of drug resistance. Nevertheless, although numerous classes of NC inhibitors have been described,[38] to the best of our knowledge no attempt has been made to select drug-resistant mutants. Thus, it is uncertain whether those inhibitors could induce mutations conferring high barrier to drug resistance.

The novel finding of the present work is, therefore, the capability of an NCp7 inhibitor class to select for resistant mutants carrying mutations at positions F6C and R32K of the *gag* gene coding for the NCp7. Interestingly, these mutations do not occur at any of the positions described above as

essential for NCp7 functions, which is consistent with the *in vitro* emergence of vital mutants whose fitness, however, remains to be investigated.

Further studies are under way with the aim of: i) confirming, through site-directed mutagenesis studies, the correlation between the F6C and R32K mutations and the development of resistance, as well as the contribution of each mutation to the resistance phenomenon; ii) the capability of resistant mutants to infect cervical mucosa explants as marker of viral fitness.

One more piece of evidence in favour of deepening the knowledge on the new class of HIV inhibitors described herein comes from the experiments carried out to comparatively investigate the direct virucidal activity of **6** and amphothericin B. Both compounds inactivate the infectivity of stock solutions of HIV-1 and HIV-2, showing strong virucidal activity at 37 °C. However, their modes of action are clearly different, 6 being unable to inactivate virion infectivity at 0° C.

In conclusion, the novel class of 1,2-benzisothiazol-3(*2H*)-one benzenesulfonamides shows promise as potential anti-HIV agents because of their structural simplicity, fast synthetic procedures and an intriguing mode of action, and deserves further insights aimed at developing new, more potent derivatives.

15.0 REFERENCES

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ABSTRACT

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