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Viral Proteins that Enhance Membrane Permeability

María Eugenia González and Luis Carrasco

1. Introduction

During the infection of cells by animal viruses, membrane permeability is modified at two different steps of the virus life cycle (Carrasco, 1995) (Figure 6.1). Initially, when the virion enters cells, a number of different-sized molecules are able to co-enter the cytoplasm with the virus particles (Fernandez-Puentes and Carrasco, 1980; Otero and Carrasco, 1987). Membrane potential is reversibly destroyed, being restored several minutes later. Endosomes are involved in the co-entry process, since inhibitors of the proton ATPase block early permeabilization even with viruses that do not require endosomal function. A chemiosmotic model has been advanced to explain the molecular basis of early membrane modification by virus particles (Carrasco, 1994). The viral molecules involved are components of virions: glycoproteins when enveloped particles are analyzed or, still unidentified, domains of the structural proteins in the case of naked viruses. Attachment of the particle to the cell surface receptor does not alter membrane permeability by itself. Inhibitors that hamper virus decapsidation, still allowing virus attachment to the cell surface, block early membrane permeabilization (Almela *et al*., 1991).

At late times of infection, when there is active translation of late viral mRNAs, the plasma membrane becomes permeable to small molecules and ions (Carrasco, 1978) (Figure 6.1). Different viral molecules may be responsible for this late enhancement of membrane permeability, including viroporins (Gonzalez and Carrasco, 2003), glycoproteins, and even proteases (Chang *et al*., 1999; Blanco *et al*., 2003). This chapter is devoted to reviewing some characteristics of membrane permeabilization by viral proteins. In addition, the methodology used to assay enhanced permeability in animal cells is described. Finally, the design of selective viral inhibitors based on the modification of cellular membranes during virus entry or at late times of infection is also discussed.

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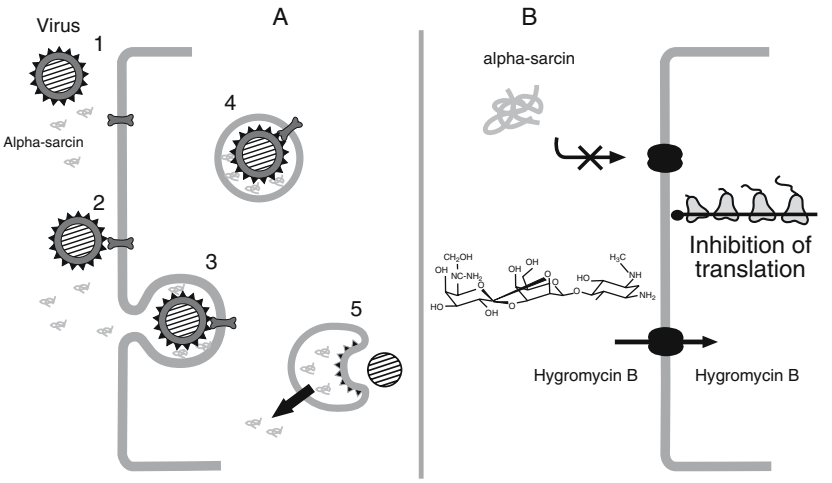

Figure 6.1. (A) Schematic representation of early membrane permeability. The different steps of virus attachment, entry, and fusion events are shown. The protein toxin alpha-sarcin co-enters endosomes in conjunction with animal virus particles. The fusion of the viral membrane with the endosome membrane induces the release of alpha-sarcin to the cytoplasm. (B) Permeabilization of the plasma membrane at late times of infection. The figure depicts the entry of the low molecular weight translation inhibitor hygromycin B through pores created at the plasma membrane. Alpha-sarcin is unable to pass through these pores.

2. Measuring Alterations in Membrane Permeability

2.1. The Hygromycin B Test

A number of hydrophilic molecules, including some antibiotics, poorly permeate through cellular membranes (Contreras *et al*., 1978; Lacal *et al*., 1980). This is the case of hygromycin B, anthelmycin, blasticidin S, destomycin A, gougerotin, and edein complex. The aminoglycoside antibiotic hygromycin B (MW 527) is produced by *Streptomyces hygroscopicus*. This hydrophilic molecule is an efficient inhibitor of protein synthesis in cell-free systems but interferes very poorly with translation in intact cells. However, the modification of the plasma membrane by viruses or by other means leads to a rapid blockade of translation (Carrasco, 1995). Concentrations of the antibiotic ranging from about 0.1–1 mM are added to the culture medium and protein synthesis is estimated by incubation with radioactive methionine for 1 hr (see Figure 6.2) (Gonzalez and Carrasco, 2001). In addition to its simplicity, the hygromycin B test has a number of advantages for assaying changes in membrane permeability. One is its great sensitivity, and another is that this test measures membrane modifications only in cells that are metabolically active. Moreover, in cultures where some cells are uninfected, hygromycin B would only enter virus-infected cells that are synthesizing proteins. The hygromycin B test has been applied with success to prokaryotic (Lama and Carrasco, 1992) and eukaryotic cells, including yeast (Barco and Carrasco, 1995, 1998) and mammalian cells (Gatti *et al*., 1998; Gonzalez and Carrasco, 2001).

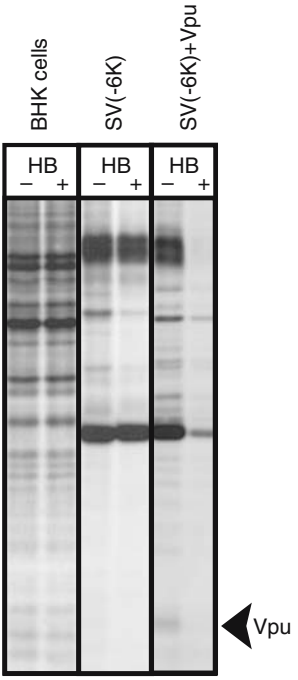

Figure 6.2. Entry of hygromycin B into BHK cells promoted by the expression of HIV-1 Vpu. Control BHK cells (left), cells infected with Sindbis virus lacking the 6K gene (SV(-6K)) (center), or with (SV(-6K)) containing the HIV-1 *vpu* gene (SV(-6K)+Vpu) (right) were treated or not with 0.5 mM hygromycin B for 10 min and protein synthesis was assayed by extended incubation with [^{35}S] Met/Cys for 1 hr. The labeled proteins were analyzed by autoradiography after SDS PAGE. For additional details see Gonzalez and Carrasco (2001).

2.2. Entry of Macromolecules into Virus-Infected Cells

Alpha-sarcin is a protein of 150 amino acid residues, which is produced by *Aspergillus giganteus* (Oka *et al*., 1990). This protein inhibits translation by modifying ribosomes in an enzymatic manner. Thus, a molecule of alpha-sarcin is able to inactivate a great number of ribosomes by hydrolysis of the A4324-G4325 phosphodiester bond in the 28S rRNA (Chan *et al*., 1983). This toxin does not enter mammalian cells because it does not attach to the cell surface and is therefore unable to cross the plasma membrane. However, alpha-sarcin efficiently interferes with protein synthesis in cell-free systems or in cells where the permeability barrier has been destroyed (Fernandez-Puentes and Carrasco, 1980). Alpha-sarcin co-enters cells in conjunction with virus particles, and is liberated to the cytoplasm (Otero and Carrasco, 1987; Liprandi *et al*., 1997). In this manner, this toxin irreversibly blocks translation several minutes after virus entry. The molecular basis of the co-entry of macromolecules with virus particles has been analyzed in detail elsewhere (Carrasco, 1994, 1995). Apart from alpha-sarcin, a number of proteins that interfere with translation, many of them of plant origin, have been described (Fernandez-Puentes and Carrasco, 1980; Lee *et al*., 1990). The release of all these toxins into cells is enhanced by virus particles. Not only proteins, but also

other macromolecules, including nucleic acids efficiently co-enter with virus particles (Cotten *et al*., 1992). However, none of these macromolecules passes into cells at late times of infection.

2.3. Other Assays to Test the Entry or Exit of Molecules from Virus-Infected Cells

Apart from the use of translation inhibitors that do not easily permeate into intact cells, a number of assays can be employed to assess modifications in membrane permeability. Amongst these assays, we can list the following.

2.3.1. Entry or Exit of Radioactive Molecules

Cells are preloaded with radioactive uridine and the exit of nucleotides can be monitored after induction of viroporin expression (Gonzalez and Carrasco, 1998). Unlike with most amino acids, the pool of uridine nucleotides is abundant in the cell interior, thus providing a convenient and sensitive assay for monitoring the exit of molecules from cells. Other tests use radioactive glucose derivatives (e.g., 2-deoxyglucose), which cannot be metabolized and accumulate in cells. The analysis of the release of radioactive compounds that are not actively transported into cells leads to the failure to measure enhanced membrane permeability.

2.3.2. Entry of ONPG and Dyes

Entry of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) into the bacterial cells can be determined very simply. This β-galactosidase substrate can be incubated with bacterial cells and production of the resulting compound can be followed by determining the absorbance at 420 nm (Lama and Carrasco, 1992). There are a number of non-vital dyes employed to characterize cell mortality. It should be noted that the entry of these compounds, in fact, determines a modification in cell membranes, which in some cases does not directly correlate with cell death. Trypan blue is a dye widely used for monitoring enhanced membrane permeability. However, this assay is not very sensitive. In addition, trypan blue staining does not discriminate between metabolically active or dead cells, as the hygromycin B tests does. Another assay employs the polyamine neurobiotin that needs specific connexin channels to enter mammalian cells (Elfgang *et al*., 1995). Permeabilization of cell membrane increases uptake of this cationic molecule. Internalized neurobiotin can be detected in paraformaldehyde-fixed cells, by fluorescence microscopy, using fluorescein isothiocyanate-conjugated streptavidin (Gonzalez and Carrasco, 1998).

2.3.3. Entry of Propidium Iodide

Propidium iodide (PI) is a DNA-intercalating compound that does not enter intact cells. However, those cells that exhibit increased membrane permeability are able to take up PI, which can be assayed by cell fluorometric analysis (Arroyo *et al*., 1995).

2.3.4. Release of Cellular Enzymes to the Culture Medium

The appearance in the culture medium of cellular enzymes is a clear indicator of cell mortality. This is the case for lactic dehydrogenase and bacterial β-galactosidase, present

outside the cells (Sanderson *et al*., 1996). Commercial kits to measure this enzymatic activity are available. The release of cellular proteins to the medium occurs at very late times of viral infection, when cells have already died. This alteration takes place at much later times after hygromycin B entry can be detected (Blanco *et al*., 2003).

3. Viral Proteins that Modify Permeability

3.1. Viroporins

Viroporins are small proteins encoded by viruses that contain a stretch of hydrophobic amino acids (Gonzalez and Carrasco, 2003). Typically, viroporins are comprised of some 60–120 amino acids. The hydrophobic domain is able to form an amphipathic α-helix. The insertion of these proteins into membranes followed by their oligomerization creates a hydrophilic pore. The architecture of this channel is such that the hydrophobic amino acid residues face the phospholipid bilayer while the hydrophilic residues form part of the pore. In addition to this domain, there are other features of viroporin structure, including a second hydrophobic region in some viroporins that also interacts with membranes. This second interaction may further disturb the organization of the lipid bilayer. These proteins may also contain a stretch of basic amino acids that acts in a detergent-like fashion. All these structural features contribute to membrane destabilization. More recently, another domain has been described in some glycoproteins and also viroporins that has the capacity to interact with membranes. This domain is rich in aromatic amino acids and is usually inserted at the interface of the phospholipid bilayer (Suarez *et al*., 2000; Sanz *et al*., 2003). This type of interaction also leads to membrane destabilization, further enhancing membrane permeability.

A number of viroporins from different viruses that infect eukaryotic cells have been reported. This group of proteins includes picornavirus 2B and 3A, alphavirus 6K, retrovirus Vpu, paramyxovirus SH, orthomyxovirus M2, reovirus p10, flavivirus p7, phycodnavirus Kcv, coronavirus E, and rhabdovirus alpha 10p. A recent review devoted to viroporins discusses the structure and function of a number of proteins of this group (Gonzalez and Carrasco, 2003), and so the details of each particular viroporin will not be reviewed in this chapter.

The main activity of viroporins is to create pores at biological membranes to permit the passage of ions and small molecules. The cloning and individual expression of viroporin genes has allowed their effects in bacterial and animal cells to be analyzed. Thus, the expression of this type of viral gene enhances the permeation of ions and several hydrophilic molecules in or out of cells (Carrasco, 1995). In addition, the purified viroporin molecules open pores in model membranes, providing a system that is amenable to biophysical analysis (Fischer and Sansom, 2002). The pore size created by viroporins allows the diffusion of different molecules with a molecular weight below about 1,000 Da.

The main step affected in animal viruses containing a deleted viroporin gene is the assembly and exit of virions from the infected cells (Klimkait *et al*., 1990; Liljestrom *et al*., 1991; Loewy *et al*., 1995; Betakova *et al*., 2000; Watanabe *et al*., 2001; Kuo and Masters, 2003). These genes are not essential for virus replication in culture cells, but the plaque size is much smaller in viroporin-defective viruses. Notably, virus entry and gene expression in viroporin-deleted viruses occur as in their wild-type counterparts. An aspect of viroporin function at the molecular level that is still not understood is the link between pore activity and virus budding.

3.2. Viral Glycoproteins that Modify Membrane Permeability

In addition to small hydrophobic viral proteins, there are other virus products that promote membrane permeabilization. This occurs with a number of virus glycoproteins (GP) that are known to increase cell membrane permeability, such as the human immunodeficiency virus gp41 (Chernomordik *et al*., 1994; Arroyo *et al*., 1995), the Ebola virus GP (Yang *et al*., 2000), the cytomegalovirus US9 protein (Maidji *et al*., 1996), the Vaccinia virus A38L protein (Sanderson *et al*., 1996), rotavirus VP7 and NS4 proteins (Charpilienne *et al*., 1997; Newton *et al*., 1997), the hepatitis C virus E1 protein (Ciccaglione *et al*., 1998), and the alphavirus E1 protein (Nyfeler *et al*., 2001; Wengler *et al*., 2003).

The architecture of some viral glycoproteins is such that upon oligomerization, the transmembrane (TM) domains may form a physical pore. In principle, two different regions of a viral fusion glycoprotein could form pores. One such region contains the fusion peptide that would create a pore in the cell membranes upon insertion (Skehel and Wiley, 1998), while the TM domain would form a pore in the virion membrane (Wild *et al*., 1994). Moreover, sequences adjacent to the TM region could have motifs designed to destabilize membrane structure (Suarez *et al*., 2000). Entry of enveloped animal viruses leads to early membrane permeabilization, which is mediated by the formation of the two pores (fusion and TM) formed by viral fusion glycoproteins. This early permeabilization induced during the entry of virions requires conformational changes of the fusion glycoproteins. By contrast, after virus replication, newly synthesized glycoproteins may affect membrane permeability when they reach the plasma membrane (Figure 6.3). This modification is achieved only by the TM domain, while the fusion peptide does not participate in this late modification. In viruses that lack the typical viroporin, its function could be replaced by these pore-forming glycoproteins, while for other viruses viroporin activity may be redundant (Bour and Strebel, 1996). In the latter case, pore formation may be generated by viral glycoproteins and viroporins (Figure 6.3). We would like to propose the possibility that pore-forming glycoproteins play a key role mainly during virus entry and, in some cases, also during virus budding, while viroporins come into action when viruses need to exit the cell.

Early membrane permeabilization is always carried out by a virion component. In the case of enveloped viruses, this early event is executed by a structural glycoprotein, which is coupled to the fusion process. An understanding of fusion at the molecular level also requires an explanation of the phenomenon of early membrane permeabilization. We have advanced the idea that viral glycoproteins involved in membrane fusion participate in the dissipation of the chemiosmotic gradient, thus providing the energy to push the nucleocapsid and neighboring macromolecules to the cell interior (Carrasco, 1994; Irurzun *et al*., 1997). Fusion glycoproteins do not simply serve to bridge the cellular and the viral membrane, but instead are designed to open pores in both membranes. This pore-opening activity may be necessary to lower membrane potential and to dissipate ionic gradients. Several chapters of this book are devoted to the detailed description of the structure and function of these glycoproteins, so we will focus our attention on viral glycoproteins that permeabilize membranes when individually expressed in cells. These membrane active proteins may exhibit this activity later on in the virus life cycle.

3.2.1. Rotavirus Glycoprotein

Rotavirus infection provokes a number of alterations in cellular membranes during infection (del Castillo *et al*., 1991). Amongst these alterations, there is an increase in the

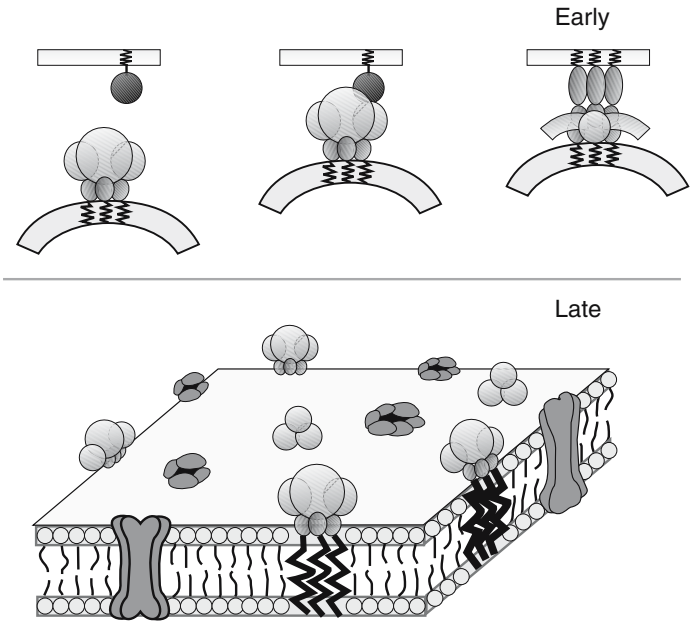

Figure 6.3. Participation of pore formation by viral glycoproteins and viroporins in membrane permeability. Early membrane permeabilization is coupled to the fusion activity of the corresponding viral glycoprotein. This fusion glycoprotein may create two pores. One is located at the target cell membrane and the other is formed by the TM domain. Late membrane permeabilization may be carried out by viroporins or by the TM domains of viral glycoproteins.

concentration of cytoplasmic calcium (Michelangeli *et al*., 1991). Several rotavirus proteins exhibit membrane-destabilizing activity. The enterotoxin NSP4 induces alterations in membrane permeability (Tian *et al*., 1994). The individual expression of the non-structural glycoprotein NSP4 has the ability to increase the concentration of cytoplasmic calcium. This increase may be mediated by activation of phospholipase C activity (Dong *et al*., 1997).

Rotavirus particles induce the co-entry of protein toxins into cells (Cuadras *et al*., 1997). At least two structural components possess the ability to permeabilize cells, including VP5 protein and VP7 glycoprotein (Charpilienne *et al*., 1997; Irurzun *et al*., 1997).

3.2.2. The HIV-1 gp41

Infection of lymphocytic human cells by HIV-1 enhances membrane permeability to ions and several compounds (Voss *et al*., 1996; Gatti *et al*., 1998). There are at least three different HIV-encoded proteins responsible for these alterations: Vpu protein, the retroviral protease, and the fusion glycoprotein gp41. Apart from the fusion peptide, there are two regions of gp41 that exhibit membrane permeability; one is located at the carboxy terminus (Arroyo *et al*., 1995; Comardelle *et al*., 1997) and another corresponds to the membrane-spanning domain (Arroyo *et al*., 1995). The C-terminus of gp41 includes two 20–30 residues, which may form cationic amphipathic α-helices, designated as lentivirus lytic peptides 1 and 2 (LLP-1 and LLP-2). Synthetic LLP-1 peptide forms pores in planar phospholipid bilayers

(Chernomordik *et al*., 1994), permeabilizes HIV-1 virions to deoxyribonucleoside triphosphates (Zhang *et al*., 1996), and induces alterations in ion permeability of *Xenopus* oocytes (Comardelle *et al*., 1997).

3.2.3. Other Viral Glycoproteins

Inducible expression of the hepatitis C virus E1 glycoprotein increases membrane permeability in bacterial cells. The ability of E1 to modify membrane permeability has been mapped to the carboxy terminus of the protein (Ciccaglione *et al*., 1998, 2001). Similar permeabilization was found with *Escherichia coli* cells that synthesize Semliki forest virus E1 glycoprotein after exposure to low pH (Nyfeler *et al*., 2001). Finally, overexpression of Vaccinia virus A38L glycoprotein produces changes in the morphology, permeability, and adhesion of mammalian cells. The potential capacity of A38L protein to form pores at the plasma membrane promotes the entry of calcium ions and PI and the release of lactic dehydrogenase into the culture medium (Sanderson *et al*., 1996).

4. Membrane Permeabilization and Drug Design

4.1. Antibiotics and Toxins that Selectively Enter Virus-Infected Cells

Different approaches have been envisaged for the design of compounds that interfere with virus replication based on modifications in membrane permeability. One such approach makes use of inhibitors of cellular or viral functions that do not permeate easily into intact animal cells. Notably, these agents selectively enter into virus-infected cells (Carrasco, 1978; Benedetto *et al*., 1980). Most of the inhibitors used thus far interfere with protein synthesis, although compounds that affect other functions could also be employed. Entry of these agents leads to the inhibition of translation specifically in virus-infected cells, leading to a profound inhibition of virus growth (Contreras *et al*., 1978; Carrasco, 1995; Gatti *et al*., 1998). Although this approach discriminates well between uninfected or virus-infected cells in culture, the high toxicity of the agents thus far assayed has hampered its use in whole animals. Perhaps future searches for less toxic compounds would make this approach amenable to application in therapy. In fact, some of the plant toxins that co-enter with virus particles have been described as being antiviral agents (Lee *et al*., 1990). Even compounds such as hygromycin B, which has been used in the veterinary field as an antibacterial agent, could also be used as an antiviral compound for rotavirus infections (Liprandi *et al*., 1997).

4.2. Viroporin Inhibitors

The paradigm of an inhibitor of a viral ion channel is amantadine (Hay, 1992) (Figure 6.4). This compound has been used as an anti-influenza agent in humans (De Clercq, 2001). The target of amantadine is the influenza-encoded protein M2. Residues 27, 30, 31, and 34 of M2 determine the amantadine sensitivity of this ion channel. A drawback of amantadine is the high doses necessary to affect influenza. The search for more effective compounds may provide a more efficacious treatment for this illness.

Compounds that interfere with the functioning of other viroporins have also been described. This is the case of amiloride derivatives that block HIV-1 Vpu activity (Ewart *et al*.,

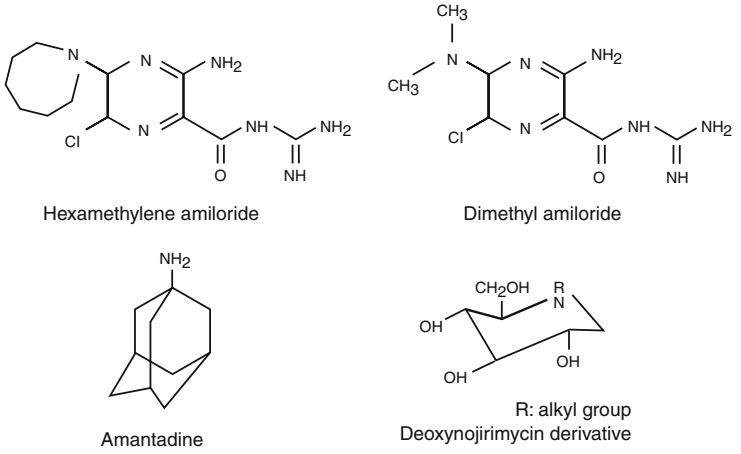

Figure 6.4. Chemical structures of several inhibitors of viroporin activity.

2002). In this manner, the production of infectious HIV-1 is reduced in the presence of this agent. Recently, long alkyl-chain iminosugar derivatives have been found to interfere with the function of the hepatitis C virus p7 protein as an ion channel (Griffin *et al*., 2003; Pavlovic *et al*., 2003). These compounds exhibited antiviral activity with bovine viral diarrhea virus, which is closely related to the hepatitis C virus (Durantel *et al*., 2001).

4.3. Antiviral Agents that Interfere with Viral Glycoproteins

Much effort has been concentrated recently on the development of antiviral agents that inhibit the fusion step of HIV. Binding of HIV gp120 to the CD4 receptor is followed by further interaction of this viral glycoprotein with the coreceptor molecules CXCR4 and CCR5. After this initial interaction, the conformation of the ectodomain of the TM glycoprotein gp41 is profoundly modified. Exposure of the fusion peptide at the amino terminus of gp41 triggers its insertion into the target cellular membrane, leading to the fusion of the viral and the cellular plasma membranes. All these steps have been used as targets for anti-HIV therapy (Cooley and Lewin, 2003). As regards the fusion step, a variety of peptide mimetic inhibitors have been developed. The pioneering work on peptide T20 has demonstrated that this compound is a potent inhibitor of gp41-induced membrane fusion. T20 exhibits antiviral activity in HIV-infected patients. The detailed mechanism of action of T20 at the molecular level is known. This peptide is homologous with 36 amino acids within the C-terminal heptad repeat region (HR2) of HIV-1 gp41. T20 competitively binds to HR1 and interferes with the formation of the six helix HR1–HR2 bundle complex necessary for membrane fusion. At present there are a great number of peptides that interfere with binding of gp120 or with gp41-induced membrane fusion and that have been tested for their anti-HIV activity and clinical efficacy. In this regard, T1249 is one of the second generation of HR-2 peptide mimetic inhibitors that consists of 39 amino acids. PRO 542 is a soluble CD4 receptor (CD4-IgG2) that binds to and neutralizes gp120 before virus binding occurs. SCH-C is an oxime–piperidine compound that is a coreceptor antagonist. This small molecule acts as an inhibitor of CCR5. MD3100 is

a non-peptidic, low molecular weight bicyclam compound that prevents interactions between CXCR4 and gp120, blocking signal transduction from CXCR4. Future research in this field will provide us with additional antiviral compounds to add to the anti-HIV armory.

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