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Pharmac. Ther. Vol. 64, pp. 215-290, 1994
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0163-7258/94 $26.00

Associate Editor: D. SHUGAR

PICORNAVIRUS INHIBITORS

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Abstract—Picornaviruses are among the best understood animal viruses in molecular terms. A number of important human and animal pathogens are members of the Picornaviridae family. The genome organization, the different steps of picornavirus growth and numerous compounds that have been reported as inhibitors of picornavirus functions are reviewed. The picornavirus particles and several agents that interact with them have been solved at atomic resolution, leading to computer-assisted drug design. Picornavirus inhibitors are useful in aiding a better understanding of picornavirus biology. In addition, some of them are promising therapeutic agents. Clinical efficacy of agents that bind to picornavirus particles has already been demonstrated.

Keywords—Picornavirus, poliovirus, antiviral agents, drug design, virus particles, viral proteases.

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Abbreviations—2'-5' A, ppp(A2'p5'A)nA; BFA, brefeldin A; BFLA1, basilomycin A1; dsRNA, double-stranded RNA; EMC, encephalomyocarditis; FMDV, foot-and-mouth disease virus; G413, 2-amino-5-(2-sulfamoylphenyl)-1,3,4-thiadiazole; HBB, 2-(2-hydroxybenzyl)-benzimidazole; HIV, human immunodeficiency virus; HPA-23, ammonium 5-tungsto-2-antimonate; ICAM-1, intercellular adhesion molecule-1; IP3, inositol triphosphate; M12325, 5-aminosulfonyl-2,4-dichorobenzoate; 3-MQ, 3-methyl quercetin; IRES, internal ribosome entry site; L protein, leader protein; RF, replicative form; RI, replicative intermediate; RLP, ribosome landing pad; SFV, Semliki forest virus; TOFA, 5-(tetradecyloxy)-2-furoic acid; VPg, viral protein bound to the genome; VSV, vesicular stomatitis virus.
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1. INTRODUCTION

The Picornaviridae family has been the object of interest of many research groups for more than 30 years. As a result, members of this virus family are among the best understood animal viruses in molecular terms. A number of excellent reviews have been published covering different aspects of picornavirology (Putnak and Phillips, 1981; Koch and Koch, 1985; Carrasco and Castrillo, 1987; Krausslich and Wimmer, 1988; Rossmann and Johnson, 1989; Sonenberg, 1990; Palmenberg, 1990; Stanway, 1990; Racaniello, 1990; Lawson and Semler, 1990; Richards and Ehrenfeld, 1990; Minor, 1990; Sarnow et al., 1990; Minor, 1992; Kirkegaard, 1992; Agol, 1993; Harber and Wimmer, 1993). Picornaviruses are structurally simple and appear as spherical particles of ~30 nm diameter that possess icosahedral symmetry. The genome is a molecule of single-stranded RNA of positive polarity. This family was first divided into four genera: Enterovirus, Cardiovirus, Rhinovirus and Aphtovirus. More recently, an additional genus, Hepatovirus, has been recognized on the basis of genome sequence and organization (Fig. 1). Some members of this group are of great importance in medicine and veterinary science, because they are pathogens of humans or of animals and, hence, of economic or ecological interest. Examples include poliovirus, the causative agent of poliomyelitis, coxsackie virus, a factor responsible for causing heart disease, and echo viruses that cause meningitis in humans, all three belonging to the Enterovirus genus. The main causative agents of common colds are rhinoviruses (genus Rhinovirus). Hepatitis A, a virus that causes hepatitis, was previously classified in the Enterovirus genus, but is now included in the Hepatovirus genus (Stanway, 1990; Harber and Wimmer, 1993). Finally, the infective agent for one of the most threatening afflictions of cattle, foot-and-mouth disease, is a member of the Aphtovirus genus. Although effective vaccines have been developed for some of these viruses, such as poliovirus or hepatitis A virus, the development of vaccines for other members of this group, such as rhinoviruses, has yet to be achieved (Minor et al., 1990; Murdin et al., 1990; Minor, 1990, 1992). Thus, it is important to develop antiviral agents to combat disease. Apart from the use of antiviral agents in this respect, these compounds play a pivotal role in molecular virology. Indeed, antiviral agents are very useful for elucidating numerous viral processes, if we can identify the exact target of the agent at the molecular level. In addition, antiviral agents provide clean and accurate tools that aid in the dissection of the complicated molecular steps of viral genome replication. Hence, antiviral agents are to molecular virology what other inhibitors are to molecular biology.

Fig. 1. Genome organization of different picornavirus species. Each protein is designated according to the actual nomenclature of picornavirus proteins. The number of nucleotides per genome and in the leader region, before the initiator AUG, are indicated. The numbers in parentheses represent the number of amino acids of each protein.
Fig. 2. Diagrammatic representation of the poliovirus genome and the proteolytic processing of poliovirus proteins. The three different types of proteolytic cleavages, polysomal, cytoplasmic and during assembly, are indicated.

Picornavirus inhibitors represent good examples of compounds that are not only useful in providing a better understanding of the biology of picornaviruses, but also in furnishing promising agents that are being assayed in clinical trials against the common cold, for instance. The large number of compounds now available for the selective blockade of different steps of the picornavirus replication cycle makes these agents invaluable tools for picornavirologists, but it is difficult to organize a comprehensive review covering all of them. Although I have tried to include as many examples as possible of the approaches used and the agents employed, inevitably, coverage will be incomplete. I apologise in advance to those whose work is not included, despite its merits.

1.1. Picornavirus Genome Organization

I shall not review here the structure of the picornavirus particle, or the different steps of its replication cycle; these topics will be covered at the beginning of each section dealing with specific picornavirus inhibitors. I shall briefly outline, instead, the structure of the genome by taking poliovirus as a representative member of this viral family.

Poliovirus was the first picornavirus and, in fact, the first animal virus with an RNA genome to be sequenced (Kitamura et al., 1981; Racaniello and Baltimore, 1981a). Soon after this achievement, infectious cDNA was obtained, thus opening new possibilities for the genetic manipulation of this important human pathogen (Racaniello and Baltimore, 1981b; Omata et al., 1984; Sarnow et al., 1990; Kirkegaard, 1992). The RNA genome is a 7,544 stretch of nucleotides, of which almost 90% encodes for proteins (Sarnow et al., 1990; Harber and Wimmer, 1993) (Fig. 2). The AUG that initiates protein synthesis is located at position 754 and is preceded by several AUGs (6–8, depending on the virus serotype), which are not recognized by the translation initiation complex (Meerovitch and Sonenberg, 1993). The initiator AUG is followed by 2209 codons that code for a polyprotein that is cleaved by virus-encoded proteases (Krausslich and Wimmer, 1988; Lawson and Semler, 1990; Palmenberg, 1990). Two termination codons are followed by 65 nucleotides, which precede a poly(A) stretch with an average length of 75 nucleotides (Yogo and Wimmer, 1972). This is genetically determined and encoded by the genome. After infection, the viral RNA contains longer poly(A) tails of 150–200 nucleotides that, presumably, are added after replication (Spector and Baltimore, 1975a, b; Spector et al., 1975). The length of the genome and the disposition of the different viral proteins varies somewhat among the different picornavirus genera, as depicted in Fig. 1. Perhaps the most remarkable variations are the presence of an
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additional L (leader) protein (Strebel and Beck, 1986) in foot-and-mouth disease virus (FMDV) and encephalomyocarditis (EMC) virus; the coding for three copies of the protein 3B (VPg), the virtual lack of protein 2A (16 amino acids) in FMDV (Stanway, 1990) and the presence of a poly(C) tract of variable length in the 5' leader region in Aphtovirus and Cardiovirus (Clarke et al., 1987; Stanway, 1990).

1.1.1. The Non-Coding Regions

The 754 nucleotides located at the 5'-terminus of the poliovirus genome have a number of functions, of which some have yet to be fully elucidated (Sarnow et al., 1990; Kirkegaard, 1992). Among these, the following may be listed: (1) This region is necessary for replication of the RNA genome. (2) At least part of the 754 nucleotides is required to initiate the synthesis of the polyprotein. (3) This region is involved in genome packaging into virion particles. (4) Poliovirus neurovirulence mutants map in this region. In addition, the 5' leader sequence may be involved in other processes required for virus replication. Thus, one of the viral proteases, protein 3C or its precursor 3CD, can interact with this region and, perhaps, regulate genome replication (Andino et al., 1990a, b). Furthermore, a poliovirus–coxsackie hybrid has been generated that contains the 220–625 nucleotides from coxsackie virus, replacing the 220–627 nucleotides of poliovirus (Semler et al., 1986). This hybrid is a ts mutant for genome replication!

One of the most salient features of the Y-end of picornavirus mRNA is that it does not contain the cap structure typical of eukaryotic mRNA (Harber and Wimmer, 1993; Meerovitch and Sonenberg, 1993). Instead, the viral RNA terminates in pUpU. Within the virion particle, or during the replication of the viral genome, this end is covalently bound, in the case of poliovirus, to a virus-encoded peptide of 22 amino acids (Flanegan et al., 1977; Lee et al., 1977). This peptide is the viral protein 3B and is also known as VPg (viral protein bound to the genome) (Nomoto et al., 1977a, b). Since picornavirus mRNA has no cap structure, the initiation of protein synthesis by these genomes is unusual (Meerovitch and Sonenberg, 1993). The initiation of translation in eukaryotic mRNAs commences by recognition of the cap structure by the initiator complex, a process involving cap-binding proteins (Kozak, 1989; Meerovitch and Sonenberg, 1993). This recognition is followed by the attachment and scanning of the 5'-end by the initiator complex, until an AUG is found in the correct context to start translation (Kozak, 1989; Cavener and Ray, 1991). In picornavirus mRNA translation, the initiator complex binds, instead, to an internal region located between nucleotides 140–630 (Pelletier and Sonenberg, 1988). In some picornaviruses, the initiator complex then scans the remaining 5'-end until the initiator AUG is found, or it might even jump or translocate directly to this AUG (Jackson et al., 1990; Meerovitch and Sonenberg, 1993). There is a pyrimide stretch UUUCCUUUU in this region, which is conserved in picornaviruses (Beck et al., 1983; Meerovitch and Sonenberg, 1993) from which nucleotides UUUCC (560–564) combined with the position of the seventh AUG located 150 nucleotides upstream of the initiator AUG are involved in the internal initiation of translation (Meerovitch et al., 1991; Meerovitch and Sonenberg, 1993).

The 65 nucleotides located between the termination codons and the poly(A) tail play a role in the replication of viral RNA (Sarnow et al., 1990). Indeed, a function of this region in the synthesis of negative-strand copies of RNA has been indicated (Sarnow et al., 1986). The presence of a pseudoknot in this region (Iizuka et al., 1987; Jacobson et al., 1993) and the implication of these structures in the replication of viral RNAs has also been considered (Pleij, 1990). The poly(A) tail plays a function in stabilizing mRNAs, and the infectivity of poliovirus RNA without this tail is reduced (Sarnow, 1989). In addition, this poly(A) can participate in the replication of viral RNA (Richards and Ehrenfeld, 1990).

1.1.2. The Coding Region

Once the ribosome starts translation at the initiator AUG, it travels through the 2209 codons of the poliovirus genome and generates a polyprotein (Wimmer et al., 1987; Harber and Wimmer, 1993). This polyprotein is not found as such in the infected cells, because it is cleaved while it is still on polysomes (Wimmer et al., 1987; Lawson and Semler, 1990). The first cleavage is
accomplished by the viral protease 2A, releasing the precursor (P1) of the capsid proteins (see the diagram depicted in Fig. 2) (Toyoda et al., 1986). Once the other viral protease (protein 3C) has been synthesized, it cleaves between P2 and P3 (Section 4.1) (Hanecak et al., 1982). Therefore, the viral proteases are active as precursors and are able to cleave themselves in cis. Indeed, once the mature proteases have been generated during infection, they are also active in trans (Krausslich and Wimmer, 1988; Palmenberg, 1990; Lawson and Semler, 1990). For more details on the proteolytic events and the specificity of these proteases, the reader is referred to several excellent reviews (Palmenberg, 1987, 1990; Krausslich and Wimmer, 1988; Lawson and Semler, 1990; Hellen and Wimmer, 1992b). Apart from the cleavages accomplished by the viral proteases 2A and 3C, a final cleavage event occurs during the assembly process on protein VP0 to generate VP4 plus VP2 (Palmenberg, 1987, 1990). This cleavage is catalyzed by VP0 itself, in conjunction with the viral RNA (Arnold et al., 1987; Palmenberg, 1987; Hellen and Wimmer, 1992b).

In the case of poliovirus, 11 mature proteins plus a different array of precursors are produced, thus making the analysis of protein synthesis in picornavirus-infected cells very complex (Jacobson and Baltimore, 1968; Summers and Maizel, 1968). Figure 3 shows the appearance of these polypeptides on two-dimensional polyacrylamide gels. The nomenclature of the picornavirus precursors and the mature proteins has been rationalized and is known as the L-4-3-4 nomenclature (Rueckert and Wimmer, 1984). As indicated in Section 1.1, some picornviruses encode for a protein, preceding the capsid proteins, that is known as L protein. Synthesis of L protein is followed by that of four capsid proteins (P1 region) and seven polypeptides involved in vegetative functions (P2 and P3 regions). These three regions in the genome, P1, P2 and P3, are distinguished according to the two proteolytic cleavages that take place on the polyprotein at the polysomal level. The mature proteins encoded in each of these regions are numbered 1, 2 and 3, followed by a letter A, B, C or D. Thus, the eleven mature proteins are known as 1A, 1B, 1C, 1D, 2A, 2B, 2C, 3A, 3B, 3C, 3D. The capsid proteins are also known by their original names: VP4 (or 1A), VP2 (or 1B), VP3 (or 1C) and VP1 (or 1D). Given the name of a viral protein, it is possible to identify its location on the genome. The precursor proteins are known by combinations of these names; for instance, precursor 3AB, 2BC or 2C3A. Although proteins 2C3A, or even 2B3A, are not found as such in infected cells, they can be generated by genetic engineering.

2. EARLY STEPS OF PICORNAVIRUS INFECTION

2.1. The Structure of Picornavirus Particles

Picornaviruses possess small virion particles whose structure at atomic resolution has been solved (Hogle et al., 1985; Rossmann et al., 1985; Luo et al., 1987; Acharya et al., 1989; Kim et al., 1989; Rossmann and Johnson, 1989; Krishnaswamy and Rossmann, 1990; Yeates et al., 1991; Grant et al., 1992; Smyth et al., 1993). The virions have a spherical shape and exhibit icosahedral symmetry (Rossmann and Johnson, 1989). They are composed of 60 copies of each structural protein VP1, VP2, VP3 and VP4, plus a copy of the genome RNA that contains one copy of the genome-bound viral protein (VPg) (Harber and Wimmer, 1993). This genomic protein is covalently attached to the 5'-end of the viral RNA through an ester bond between the tyrosine present in VPg and the 5'-OH of the last nucleotide in the viral RNA (Lee et al., 1977). The virion is composed of 60 identical asymmetric units, each formed by a copy of each of the structural proteins, although only three (VP1, VP2 and VP3) are on the virion surface, with VP4 being located internally (Hogle et al., 1985; Rossmann and Johnson, 1989).

Determination of the structures of picornavirus proteins revealed that the virion proteins VP1, VP2 and VP3 have different amino acid sequences, but similar tertiary structures (Hogle et al., 1985; Rossmann et al., 1985; Acharya et al., 1989; Rossmann and Johnson, 1989; Grant et al., 1992). This tertiary structure is also shared by the single protein of the capsid of many plant RNA viruses, suggesting that a kind of basic structure or “brick” can exist within different virion particles (Rossmann and Johnson, 1989). The protein fold that gives rise to this basic brick is depicted in Fig. 4. It is composed of two four-stranded β-sheets and is quite similar in different viruses (Hogle et al., 1985; Rossmann et al., 1985; Acharya et al., 1989; Rossmann and Johnson, 1989). The minimum number of amino acids required to form a β-barrel is ~150 (Rossmann and Johnson,
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1989). There may be insertions of variable size between the strands at the cage of the traquetoid. VP1, VP2 and VP3 contain a common eight-stranded antiparallel β-barrel motif. Their amino termini intertwine to link them, thus forming a network in the interior of the capsid. Five VP3 amino termini form a five-stranded, helical β-cylinder in the virion interior (Hogle et al., 1985;

Fig. 3. Two-dimensional gel electrophoresis of [35S]labelled proteins from uninfected (panel A) or poliovirus-infected (panel B) HeLa cells. The cells were labelled during one hour (4–5 hr p.i.) in methionine-free medium (Urzainqui and Carrasco, unpublished results).
Rossmann et al., 1985; Acharya et al., 1989; Rossmann and Johnson, 1989). VP4 is myristoylated at the amino terminus (Chow et al., 1987; Paul et al., 1987; Urzainqui and Carrasco, 1988). This myristoylate moiety lies inside the virion coat near the β-cylinder (Rossmann and Johnson, 1989). On the surface of each protomer there is a depression of 25 Å depth, which is absent in aphthovirions (Hogle et al., 1985; Rossmann et al., 1985; Acharya et al., 1989; Rossmann and Johnson, 1989). This depression or “canyon” has been implicated in the interaction of virion particles with receptors; neutralizing antibodies cannot enter into this depression (Rossmann and Johnson, 1989; Rossmann, 1989b; Olson et al., 1993a) (Fig. 5).

2.2. Picornavirus Receptors

Viral infection commences by the recognition and attachment of virion particles to specific molecules located on the surface of cells, the so-called receptors (Dales, 1973; Dimmock, 1982; Lentz, 1990). The chemical nature of the receptor molecules varies, depending on the species of virus that infects the cells, but many identified receptors are proteins and, more particularly, glycoproteins (Dales, 1973; Dimmock, 1982; Lentz, 1990). In principle, a single virus species can use different receptor molecules on a single cell type or on different kinds of cells. In addition, different virus species can use the same receptor to land on the cell that is to become infected (Dales, 1973; Dimmock, 1982; Lentz, 1990). The presence of receptors on the cell surface determines, in many instances, the viral tropism, i.e. the susceptibility of a given set of cells in the organism to infection by a particular virus (Dales, 1973; Dimmock, 1982; Lentz, 1990). In addition, the
interaction of viruses with cell surface receptors is one of the targets through which viral infection might be blocked (Lentz, 1990).

In the case of picornaviruses, the receptor molecules for poliovirus and rhinovirus have been identified, the genes encoding these glycoproteins cloned and their protein products expressed and characterized (Greve et al., 1989; Mendelsohn et al., 1989; Staunton et al., 1989; Tomassini et al., 1989). Both receptors belong to the superimmunoglobulin family of proteins (see reviews by Racaniello, 1990; Harber and Wimmer, 1993). The poliovirus receptor is composed of three immunoglobulin-like domains located on the surface of the cell, a hydrophobic transmembrane domain that anchors the receptor to the membrane, and a cytoplasmic C-terminal region (Mendelsohn et al., 1989; Koike et al., 1990) (Fig. 6).

The intercellular adhesion molecule-1 (ICAM-1) is the cellular receptor for rhinoviruses (Staunton et al., 1989, 1990). This glycoprotein binds the lymphocyte function-associated antigen 1, an interaction involved in the immunological response (Staunton et al., 1989, 1990). The receptor molecule ICAM-1 is composed of five immunoglobulin-like extracellular domains, a transmembrane domain of hydrophobic nature, and a short C-terminal cytoplasmic domain (Staunton et al., 1989, 1990). Large amounts of truncated ICAM-1 lacking the hydrophobic and cytoplasmic domains have been obtained (Marlin et al., 1990). These truncated forms are soluble and interact with rhinovirus particles (Marlin et al., 1990; Hoover-Litty and Greve, 1993; Olson et al., 1993a). The structure of the truncated ICAM-1 complexed with rhinovirus particles has been determined using cryoelectron microscopy, indicating that the receptor binds into the canyon (Olson et al., 1993a, b).

The availability of cDNA clones coding for the poliovirus receptors made possible an analysis of the structure of the relevant gene and the transcripts derived from it in different cell lines (Koike et al., 1990, 1991a; Morrison and Racaniello, 1992). The poliovirus receptor gene contains eight exons, and its transcription produces a number of splice variants of mRNAs (Koike et al., 1990). A duplication of the poliovirus gene has been found in monkeys, the two genes being encoded at different loci of the genome (Koike et al., 1992). Mouse L cells are infected by poliovirus when transfected with the gene encoding the poliovirus receptor (Mendelsohn et al., 1989). Transgenic mice have been generated carrying the poliovirus receptor gene (Ren et al., 1990; Koike et al., 1991b; Ren and Racaniello, 1992). These mice are susceptible to poliovirus and could constitute a good animal model to test the efficacy of poliovirus vaccines and the antiviral activity of poliovirus inhibitors in animals.
The binding of virion particles to their receptors induces conformational changes in some virion proteins that could trigger the interaction of these proteins with the membrane (Frick and Hogle, 1990; Hoover-Litty and Greve, 1993). Subsequently, the translocating activity of some of the virion proteins will be put in motion, thus inducing the passage of the virion through the lipid bilayer (Carrasco et al., 1993; Pérez and Carrasco, 1993). Some virion proteins are, therefore, endowed with translocation activity similar to that displayed by some domains of polypeptide toxins (Carrasco et al., 1993). However, this translocation capacity has not yet been assigned to any picornavirus protein.

In the model indicated above, receptor molecules are regarded as playing an active role during virus entry (Frick and Hogle, 1990; Carrasco et al., 1993; Hoover-Litty and Greve, 1993). The receptor binds the particle to the cell surface and this binding induces, in the virions, conformational changes that are required to uncoat the virion particle (Moore et al., 1990; Hoover-Litty and Greve, 1993) (see Section 2.3). This process is required to allow penetration of the membrane and entry to the cytoplasm (Frick and Hogle, 1990; Hoover-Litty and Greve, 1993). In agreement with this is the finding that binding of opsonized poliovirus to mouse L cells does not lead to infection (Mason et al., 1993), indicating that the simple attachment of poliovirus to the cell surface does not suffice to initiate infection. Thus, the viral receptor in the cell plays a role beyond attachment (Mason et al., 1993). Although lowering the pH in endosomes can contribute to the conformational changes that occur in virions, this process is not necessary in the case of poliovirus (Pérez and Carrasco, 1993) (see Section 2.3). Even in some enveloped viruses that have recently been investigated, binding of enveloped virions to receptors also induces conformational changes in the viral glycoproteins that are responsible for membrane fusion (Moore et al., 1990; Meyer et al., 1992). In principle, low pH would not be required to induce the conformational changes that lead to membrane fusion in the case of enveloped viruses, but would provide a proton-motive force to help the virion particle to trespass the membrane (Carrasco et al., 1993).

2.3. Entry and Uncoating of Picornaviruses

The mechanism used by animal viruses to enter cells and to deliver their genomes into the cytoplasm is a subject of intensive research (Hoekstra and Kok, 1989; Marsh and Helenius, 1989). Animal viruses containing lipid membranes accomplish this task by fusion of the external envelope with cellular membranes, involving either the plasma membrane, as occurs with Sendai virus, herpesvirus or vaccinia virus, or the endosomal membrane, as is the case of semliki forest virus (SFV), vesicular stomatitis virus (VSV) or influenza virus (Hoekstra and Kok, 1989; Marsh and Helenius, 1989). In the latter case, fusion is promoted by conformational modifications of the viral glycoproteins after receptor binding and acidification of endosomes (Wiley and Skehel, 1987; White, 1990). Prevention of this acidification by lysosomotropic agents leads to inhibition of infection by SFV, VSV or influenza virus (Hoekstra and Kok, 1989). Models that explain the entry of animal virus particle devoid of a lipid envelope, such as picornaviruses, are more scarce (Hoekstra and Kok, 1989; Marsh and Helenius, 1989). In principle, these viruses could pass directly through the plasma membrane after receptor binding. Some evidence supports this mode of entry for certain viruses, such as adenoviruses (Brown and Burlingham, 1973), poliovirus (Dunnebacke et al., 1969), rotaviruses (Kaljot et al., 1988) and reovirus subviral particles (Borsa et al., 1979). Alternatively, the viral particles can be internalized by receptor-mediated endocytosis and then cross the endosomal membrane (Everaert et al., 1989; Marsh and Helenius, 1989; Kronenberger et al., 1992). In both cases, the viral genome must pass across a lipid membrane, aided by the virion proteins. Most evidence indicates that the majority of naked animal virus particles follow the receptor-mediated endocytosis pathway to infect cells (Marsh and Helenius, 1989). Whether some of these viruses need a low pH step to trigger the crossing of the endosomal membrane, or enter cells by a pH-independent mechanism, remains open to debate (Brown and Burlingham, 1973; Madshus et al., 1984c; Svensson, 1985; Sturzenbecker et al., 1987; Gromeier and Wetz, 1990; Kronenberger et al., 1991; Varga et al., 1991). Two major questions, therefore, remain to be answered with respect to the mechanism of picornavirus entry into cells: Is a given picornavirus
species internalized through endosomes in a particular cell type?, or do endosomes need to be acidified for the virus to enter cells?

Attachment of poliovirus and its internalization in endosomes leads to conformational changes in the virion particle, particularly in VP1 (Frick and Hogle, 1990), that allow interaction with the membrane and aid the insertion of the myristoylated protein VP4 into the membrane (Lonberg Holm et al., 1975; Guttman and Baltimore, 1977; Fricks and Hogle, 1990). This insertion not only destabilizes the particle, but may also allow interaction with the membrane of some moieties of other viral proteins (Lonberg Holm et al., 1975; Guttman and Baltimore, 1977; Fricks and Hogle, 1990) (Fig. 7). It can be speculated that the insertion of the virion proteins into the membrane would open a pore through which the genome is delivered. Alternatively, energy may be required in the form of ATP, or as the proton-motive (membrane potential and/or pH gradient) generated in endosomes by the activity of the proton-ATPase pump, that could be used to drive the nucleocapsid through the lipid barrier of the membrane (Carrasco et al., 1993).

A simple mechanistic model of viral particles opening pores in the membrane through which the genome passes is, however, inconsistent with the fact that the uncoating per se can occur in vitro in the presence of receptors (Hoover-Litty and Greve, 1993), whereas the entry of the genome into the cytoplasm requires a metabolically active cell (Hoekstra and Kok, 1989; Marsh and Helenius, 1989). The presence of ion channels in icosahedral virus particles (Kalko et al., 1992) may indicate that insertion of these capsids into membranes opens the ion channels that are required to dissipate the ionic gradients between endosomes and the cytoplasm.

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**Fig. 7.** Two potential routes for picornavirus entry into cells and the co-entry of the toxin α-sarcin. 1: the viral particle binds to its receptor and follows the endosomal route. 2: direct entry through the plasma membrane. The proton gradient generated by the vacuolar proton-ATPase is used by the virion proteins to translocate α-sarcin through the vacuolar membrane.
2.4. Early Membrane Permeabilization Promoted by Virus Particles

One approach towards an understanding of the mechanisms by which animal viruses traverse the cellular membrane is to elucidate, in molecular terms, how the co-entry of protein toxins, or other macromolecules, and viral particles is achieved (Yamaizumi et al., 1979; Fernández-Puentes and Carrasco, 1980; FitzGerald et al., 1983; Carrasco et al., 1989). The infection of susceptible cells by picornaviruses leads to profound changes in membrane permeability at two well-defined periods of the infection cycle: (1) early during infection when the virus enters the cells or (2) at the beginning of the late phase, before the onset of viral macromolecular synthesis takes place (for a review, see Carrasco et al., 1989). This late modification of membrane permeability is also known as “late membrane leakiness” (Carrasco, 1978; Carrasco et al., 1989). The molecular basis underlying both phenomena differs. Thus, only virion particles are necessary to accomplish the increase in membrane permeability early during infection (Fernández-Puentes and Carrasco, 1980; Carrasco, 1981), whereas viral gene expression is necessary for the development of the late membrane leakiness (Carrasco, 1978; Contreras and Carrasco, 1979). Selective compounds have been developed that block the replication of animal viruses during early and late modifications of the membrane (Carrasco and Vazquez, 1983).

Fernández-Puentes and Carrasco (1980) first observed that viral particles permeabilized cells to protein toxins that are otherwise unable to cross the membrane, because there are no receptors for them. The proteins are efficiently delivered into the cytoplasm shortly after addition of picornavirus to the medium. Indeed, almost 100% of the cells become permeabilized in a few minutes (Otero and Carrasco, 1987b; Lee et al., 1990), suggesting that the viral particle contains a component that not only promotes the entry of the viral nucleocapsid into the cell, but also directs other macromolecules that are not physically bound to the particles towards the cytoplasm (Figs 7 and 8).

We now know that different protein toxins can cross the membrane in the presence of animal virus particles, and that other proteins, such as luciferase or horse-radish peroxidase (Otero and Carrasco, 1987b), and polysaccharides (González et al., 1987) are also delivered into cells by these particles (Fig. 8). No infectious virions are required for early membrane permeabilization to occur, because this phenomenon takes place with UV-inactivated (but not heat-inactivated) virions (Carrasco, 1981; Otero and Carrasco, 1987b; Carrasco et al., 1989). More recently, the permeabilization of cells by adenovirus particles has been used to introduce DNA into cells. In a series of elegant experiments, Birnsteil and colleagues have shown that plasmids can bind to cells when they are complexed with transferrin-polylysine molecules (Zenke et al., 1990; Wagner et al., 1991). The entry of these complexes is enhanced by more than 2000-fold when adenovirus particles are present in the culture medium. In this fashion, more than 90% of the cells express the transfected gene (Cotten et al., 1992; Wagner et al., 1992a, b; Zatloukal et al., 1992).
2.4.1. Mechanism of the Early Membrane Permeabilization

We are starting to understand the exact mechanism by which the early membrane permeabilization of membranes, induced by viral particles, occurs. Certainly, receptor binding of poliovirus particles and virion uncoating are necessary for this process to take place (Almela et al., 1991). It has been speculated that the delivery of protein toxins by animal viruses involves the disruption of endosomes (FitzGerald et al., 1983; Carrasco et al., 1989), but other more selective mechanisms are possible. α-Sarcin has no effect on protein synthesis when added to uninfected HeLa cells, whereas the toxin efficiently blocks translation when added together with virion particles (Fernández-Puentes and Carrasco, 1980; Otero and Carrasco, 1987b). This co-internalization of the protein toxin by poliovirus is potently blocked by bafilomycin A1 (BFLA1) (see Section 3.3.2), at concentrations that prevent infection with SFV or VSV (Pérez and Carrasco, 1993).

These results indicate that an active vacuolar proton-ATPase is necessary for efficient delivery of α-sarcin into the cytoplasm mediated by poliovirus particles. We may conclude that most, if not all, of the toxin delivered into the cytoplasm comes from endosomal entry, because the inhibition of HeLa cells by α-sarcin was totally prevented by inhibiting the vacuolar proton-ATPase with BFLA1 (Pérez and Carrasco, 1993). A model of poliovirus entry illustrating the co-entry of α-sarcin and the site of action of different inhibitors is discussed in Section 3.4. Poliovirus enters from endosomes into the cytoplasm. The endosomal membrane remains intact because its rupture would lead to the eruption of the endosomal content, including α-sarcin, into the cytoplasm. Although poliovirus permeabilizes cells to α-sarcin and this event occurs in endosomes, it is conceivable, although unlikely, that the virus has already released its genome into the cytoplasm after binding to the cell surface receptor, and that the virion proteins present in the membrane and internalized in endosomes are the ones that control permeabilization to α-sarcin (Fig. 7).

Thus, the simple idea that enveloped animal viruses enter cells only by fusion between two membranes (Marsh and Helenius, 1989) is perhaps untenable, because such a model does not explain how the co-internalization of other macromolecules takes place (Yamaizumi et al., 1979; FitzGerald et al., 1983; Carrasco et al., 1989; Lee et al., 1990). Perhaps the proton-motive force can be used to drive the viral nucleocapsid and other macromolecules through the lipid barrier of the membrane. Virion proteins may couple the energy liberated by ATP hydrolysis or the movement of protons and other ions into the cytoplasm in favour of its gradient to the translocation of the genome or protein toxins in the same direction (Carrasco et al., 1993).

3. COMPOUNDS THAT BLOCK EARLY STEPS OF INFECTION

3.1. Compounds that Bind to Virus Particles

As indicated in Section 2.1, on the surface of virion particles of rhinoviruses and poliovirus, there is a canyon crossing each protomer (Rossman, 1989b; Rossmann and Johnson, 1989; Harber and Wimmer, 1993). Below this canyon there is a hydrophobic pocket within the VP1 β-barrel, where a number of compounds that block virion uncoating bind (see reviews by Diana et al., 1985c; Rossmann, 1989a; Rossmann and Johnson, 1989; McKinlay et al., 1992) (Fig. 5). This binding increases protein rigidity and stabilizes the particle in such a manner that uncoating cannot proceed (Rossmann, 1988; Chapman et al., 1991; Gruenberger et al., 1991; McKinlay et al., 1992) (Figs 5 and 9). Thus, these uncoating inhibitors act as “staples” on virion particles, stabilize them and block the disassembly of viruses.

3.1.1. WIN Compounds

The most refined studies on uncoating inhibitors have been carried out on a series of compounds developed by Sterling-Winthrop that are collectively referred to as WIN compounds (Fig. 10) (Rossmann, 1989a; McKinlay et al., 1992). Current knowledge on the interaction of these antiviral agents with rhinovirus particles has laid the groundwork for computer-assisted drug design (Diana et al., 1993).

One of the earliest steps during picornavirus uncoating is the formation of “A particles”, which
have a sedimentation coefficient of 125 S and have lost VP4 (Lonberg Holm et al., 1975; Guttman and Baltimore, 1977). These particles are formed after infection of cells by picornaviruses or in vitro by exposure of virions to various treatments, e.g. altering the ionic conditions or the pH (Giranda et al., 1992). The formation of these particles in vivo and in vitro is blocked by WIN compounds (Fox et al., 1986; Zeichhardt et al., 1987; Gruenberger et al., 1991).

Studies with human rhinovirus particles at atomic resolution (Rossmann et al., 1985) have aided the elucidation of the mode of interaction of a number of WIN compounds with these particles (Smith et al., 1986; Badger et al., 1988, 1989; Rossmann, 1988, 1989a; Kim et al., 1989, 1993; Diana et al., 1990; McKinlay et al., 1992). The binding site of these antiviral agents is in a hydrophobic pocket located beneath the canyon floor (Smith et al., 1986; Rossmann, 1989a; Diana et al., 1990, 1993; Chapman et al., 1991; McKinlay et al., 1992; Kim et al., 1993; Diana and Mallamo, 1993) (see Figs 5 and 9). Binding of the drug to this pocket induces conformational changes in the floor of the canyon, causing it to be pushed upwards (Pevear et al., 1989). This deformation can interfere with the binding of some rhinovirus serotypes with receptors (Pevear et al., 1989), although other rhinovirus serotypes and poliovirus are not inhibited by WIN compounds with respect to their interaction with cell surface receptors (Fox et al., 1986; Zeichhardt et al., 1987; Almela et al., 1991). Thus, some rhinovirus serotypes would be blocked at both the binding and uncoating steps (Shepard et al., 1993), whereas inhibition of other picornaviruses by these drugs would be at the uncoating step only (Fox et al., 1986; Zeichhardt et al., 1987; Pevear et al., 1989; Almela et al., 1991).

Rhinovirus 1A and polioviruses, in contrast with other rhinoviruses, bind a cofactor of lipidic nature in the pocket underneath the canyon floor (Filman et al., 1989; Yeates et al., 1991; Kim et al., 1993). This cofactor is displaced after binding of WIN compounds, without alteration to the floor of the canyon (Kim et al., 1993). This observation may explain why attachment of virion particles in the case of rhinovirus 1A and poliovirus is not blocked by WIN compounds (Kim et al., 1989).

A number of clinical trials against common colds, using compounds that bind to rhinovirus particles and block uncoating, have been carried out (Tyrrell, 1984; Barrow et al., 1990; McKinlay et al., 1992; Diana et al., 1993). Although results with dichloroflavan, Ro 09-0410 and 44,081 RP have been disappointing, recent trials with WIN 54954, R 77975 and R 61837 are more promising (see below). One of the major problems in the therapeutic treatment of common colds is to reach drug levels in the nasal epithelium that are sufficiently high to block viral growth (Reed, 1980; Tyrrell, 1984). WIN 51711 (disoxaril) was one of the first WIN compounds to be evaluated therapeutically (see below). The development of new derivatives with a broader spectrum of action and increased potency against rhinovirus prompted their clinical evaluation. WIN 54954 was not effective against rhinovirus when administered orally to a group of volunteers (Turner et al., 1993). However, a prophylactic trial against the common cold caused by coxsackie A21 virus demonstrated the oral efficacy of this compound (McKinlay et al., 1992; Schiff et al., 1992).
3.1.2. Arildone and Disoxaril

Arildone (WIN 38020) and disoxaril (WIN 51711) (Fig. 10) also belong to the group of WIN compounds. Arildone (4-6-(2-chloro-4-methoxyphenoxy)hexyl-3,5-heptanedione) is a selective inhibitor of poliovirus uncoating (McSharry et al., 1979; Caliguiri et al., 1980; McSharry and Pancic, 1982; Eggers and Rosenwirth, 1988; Everaert et al., 1989). Arildone and some related derivatives block the replication of several RNA and DNA viruses, including herpes simplex virus types 1 and 2 (Diana et al., 1977a, b; Kuhrt et al., 1979), cytomegalovirus, SFV and VSV (Kim et al., 1980). Administration of radioactive arildone to mice, dogs and monkeys by various routes indicates that it is extensively metabolized (Benziger and Edelson, 1982). Arildone and the 3,5-dimethoxy isoxazole are active when administered orally in mice inoculated intracerebrally with poliovirus type 2 (Diana et al., 1985a). The 3,5-dimethoxy isoxazole is more active against rhinovirus type 2 in cultured cells than is 4,6-dichloroflavan (Diana et al., 1985a).

Disoxaril, 5-7-4-(4,5-dihydro-2-oxazolyl)phenox heptyl-3-methylisoxazole is a broad spectrum antipicornaviral agent (Diana et al., 1985a, b; McKinlay, 1985; Otto et al., 1985). Oral administration of WIN 51711 was effective against echovirus and prevented poliovirus-induced paralysis and death in mice (McKinlay, 1985; McKinlay and Steinberg, 1986; McKinlay and Rossmann, 1989). The W-2 strains of poliovirus type 2 can cause persistent infections in the CNS of immunosuppressed mice. Disoxazil treatment of these mice significantly decreased the incidence of disease and induced a rapid clearance of the virus from the CNS (Jubelt et al., 1989). Unfortunately, side effects appear in patients when high oral doses of this compound are given (Diana et al., 1993).

3.1.3. R 77975 (Pirodavir) and R 61837

Both of these compounds belong to a series of pyridazinamine analogues synthesized by Janssen Research Foundation (Fig. 10). They block the replication of different rhinovirus serotypes by stabilizing the viral particle and inhibiting uncoating (Andries et al., 1988; Rombaut et al., 1991). Internalization of poliovirus particles into cells is not affected by R 78206, whereas uncoating and release of these particles into the cytosol are blocked (Rombaut et al., 1991; Ofori-Anyinam et al., 1993). Assuming that these agents directly inactivate viral particles (Andries et al., 1988) and that mutants resistant to R 61837 are cross-resistant to other antiviral agents, including WIN 51711 (Andries et al., 1989), it is likely that these agents can bind into the same hydrophobic pocket, located beneath the canyon of human rhinoviruses, that is used by WIN compounds (Chapman et al., 1991). Direct evidence for this interaction has been obtained by X-ray crystallographic analysis of human rhinovirus 14 complexed with R 61837 (Chapman et al., 1991). This agent binds towards the pocket entrance, but fails to occupy the end of the pocket (Chapman et al., 1991). It has been suggested that adding a tail to R 61837 to fit into the end of the pocket would improve the drug's activity against rhinovirus 14 (Chapman et al., 1991). Clinical efficacy against the common cold was first demonstrated for R 77975 (pirodavir) (Hayden et al., 1992). Administration of R 61837, applied as a nasal spray with hydroxy-β-cyclodextrin as a vehicle, to human volunteers infected with rhinovirus 9 shows efficacy when given prophylactically, but not when used therapeutically (Al-Nakib et al., 1989; Barrow et al., 1990).

3.1.4. SCH 38057

SCH 38057 (1f6-(2-chloro-4-methoxy)-hexyl]imidazole hydrochloride) (Fig. 10) is a water-soluble antiviral agent that binds irreversibly to enterovirus and rhinovirus particles (Zhang et al., 1993). The structure of rhinovirus 14 complexed with SCH 38057 shows that this compound binds at the innermost end of the hydrophobic pocket of VP1, leaving the entrance unoccupied. This interaction induces conformational changes involving a stretch of at least 36 amino acids of VP1 and VP3 (Zhang et al., 1993).
3.1.5. *Phenoxy(pyridine)carbonitriles*

2-(3,4-Dichlorophenoxy)-5-nitrobenzonitrile (MDL-860) (Fig. 11) exhibited antipicornavirus activity *in vitro* (Powers *et al.*, 1982; Torney *et al.*, 1982). A number of phenoxybenzenes and phenoxypyridines were synthesized and evaluated for antiviral activity (Kenny *et al.*, 1985). Among these compounds, 2-(3,4-dichlorophenoxy)-5-nitrobenzonitrile was found to have a broad-spectrum antiviral activity (Kenny *et al.*, 1985). The best antipicornavirus activity was found with 6-(3,4-dichlorophenoxy)-3-(ethylthio)-2-pyridine carbonitrile (DEPC) (Kenny *et al.*, 1985). The uncoating of photosensitized coxsackie virus A21 was reduced by this compound (Kenny *et al.*, 1985). Furthermore, DEPC has recently been shown to block the uncoating step in poliovirus (González *et al.*, 1990a; Almela *et al.*, 1991).

3.1.6. *MDL 20,610*

A series of 2-phenyl-2H-pyano[2,3-b]pyridines has been synthesized (Kenny *et al.*, 1986), and some of them (MDL 20,610, MDL 20,646 and MDL 20,957) (Fig. 11) showed potent antirhinovirus activity (Kenny *et al.*, 1986). The 3',4'-dichloro substitution, coupled with a 6-methylsulfonyl, 6-bromo or 6-chloro, is required for strong inhibition, and these compounds inhibit rhinovirus growth by 99% at concentrations of 4 ng/mL (Kenny *et al.*, 1986). Studies of their mode of action indicated that MDL 20,610 binds directly to rhinovirus particles and blocks uncoating (Kenny *et al.*, 1988).
3.1.7. 4,6-Dichloroflavan (BW683C and BW4294)

A number of flavan derivatives (Fig. 11) were synthesized and assayed against rhinovirus type 1B (Bauer et al., 1981; Tisdale and Selway, 1983). At a concentration of 7 nM, 4,6-dichloroflavan (BW683C) blocked the replication of this rhinovirus in cultured cells, making this compound, at that time, the most active inhibitor of rhinovirus in culture systems (Tisdale and Selway, 1983). More recently, 3,5-dimethoxy isoxazole was shown to be more active than BW683C (Diana et al., 1985a). Clinical trials with BW683C, given orally or intranasally, failed to protect from rhinovirus infections, despite the fact that the drug achieved good blood levels when given orally. However, it could not be found in nasal secretions (Phillpotts et al., 1983a; Al-Nakib et al., 1987). Radioactive BW683C binds to rhinovirus particles and acts on an early step during infection (Bauer et al., 1981; Tisdale and Selway, 1983). Several 4,6-dicyanoflavans based on BW683C have now been synthesized and give good inhibition not only of rhinoviruses, but also of other picornaviruses (Conti et al., 1990; Desideri et al., 1990). Finally, 1-anilino-9-benzyl-2-chloropurines, containing a lipophilic para substituent, are good inhibitors of rhinovirus 1B (BW4294) (Kelley et al., 1990).

3.1.8. Ro 09-0410 and Ro 09-0696

Ro 09-0410 (4'-ethoxy-2'-hydroxy-4,6'-dimethoxychalcone) (Fig. 11) is an active compound against rhinoviruses and binds to the viral capsid (Ishitsuka et al., 1982a, b; Ninomiya et al., 1984,
1985, 1990). Radioactive chalcone (Ro 09-0410) binds specifically to rhinovirus particles and this binding is inhibited by BW683C or RMI-15,731 (Ninomiya et al., 1985; Ishitsuka et al., 1986). Of 53 rhinovirus serotypes tested, 46 were inhibited by Ro 09-0410 (Ishitsuka et al., 1982a). Mutant rhinoviruses resistant to chalcone were cross-resistant to BW683C or RMI-15,731 (Ishitsuka et al., 1986). These results indicate that the three compounds had a similar mode of action based on their binding to virion particles. Several analogues of Ro 09-0410 have been synthesized, some of them being 10 times more active than the parent compound (Ninomiya et al., 1990). Administration of 1–2 ng/mL Ro 09-0696 is sufficient to significantly reduce rhinovirus replication in cultured cells (Ninomiya et al., 1990). These analogues bind to the same site on the virus particles as Ro 09-0410 and have a similar mode of action (Ninomiya et al., 1990).

3.1.9. 44081 RP

44081 RP, 2-(1,5,10,10a-tetrahydro-3H-thiaxolo 3,4-fl-isoquinolin-3-ylidine) amino-4-thiazole acetic acid (Fig. 12), shows selective antirhinovirus activity (Zerial et al., 1985) and is active at concentrations lower than those that cause cytotoxic effects (125 μg/mL). The compound was not virucidal and acted on an early step in infection (Zerial et al., 1985) by blocking the uncoating of virion particles (Alarcón et al., 1986).

3.1.10. Rhodanine

2-Thio-4-oxothiazolidine (rhodanine) (Fig. 12) selectively inhibits the uncoating of echovirus type 12 (Eggers, 1977). The adsorption of virions to cells, and their subsequent entry into the cytoplasm, are not affected by this compound. Other picornaviruses and RNA- or DNA-containing viruses are not susceptible to inhibition by rhodanine. The compound has no deleterious effects on cell morphology or cellular macromolecular synthesis (Eggers, 1977; Eggers et al., 1979; Rosenwirth and Eggers, 1979) and protects the virion from both alkaline degradation (Eggers, 1977) and heat-inactivation (Rosenwirth and Eggers, 1979). Rhodanine apparently blocks uncoating, since the compound prevents the shut-off of host protein synthesis induced by echovirus (Rosenwirth and Eggers, 1977). It is well established that inhibition of host translation by picornaviruses requires translation of the input genomic RNA (Carrasco and Castrillo, 1987), a step that does not take place in the presence of uncoating inhibitors.
3.1.11. **RMI-15,731**

RMI 15,731 (1-[5-tetradecyloxy-2-furanyl]-ethanone) (Fig. 12) directly inactivates rhinovirus particles (Ash et al., 1979). The mode of action of this agent has still not been investigated in detail. It is possible that the compound blocks uncoating (Ninomiya et al., 1985), but it may have other effects because it was active if cells were pretreated and then washed, or if the agent was added 6 hr after infection (Ash et al., 1979). The chemical structure of RMI 15,731 is similar to that of 5-(tetradecyloxy)-1-furoic acid (TOFA), an inhibitor of poliovirus genome replication (see Section 6.11).

3.1.12. **2-Thiouracil and Methylthiopyrimidine (S-7)**

There are several agents that interact with picornavirus particles and stabilize them against heat-inactivation and/or decapsidation. This is the case for l-cystine, but not for other amino acids (Pohjanpelto, 1958), glutathione (Fenwick and Cooper, 1962), 2-thiouracil (Steele and Black, 1967) and the methylthiopyrimidine derivative S-7 (Fig. 12) (Yamazi et al., 1970; La Colla et al., 1972; Lonberg Holm et al., 1975). Treatment of poliovirus with 1 mM 2-thiouracil resulted in direct inactivation of infectivity. Perhaps oxidized forms of this compound react with sulfhydryl groups of capsid proteins, inactivating them (Steele and Black, 1967). S-7 inhibits the thermal inactivation of poliovirus particles and their decapsidation in HeLa cells (Lonberg Holm et al., 1975).

3.2. **Receptor Blockade and Soluble Receptors**

Inhibition of virus attachment to its receptor results in a block of viral growth (Lentz, 1990). This inhibition can be achieved by several means. One of them is to use antibodies against viral particles that block their interaction with receptors. Another approach is to antagonize the receptor by antibodies directed against the receptor itself (Minor et al., 1984; Colonnio et al., 1986; Minor, 1990, 1992). Application of this latter approach in human volunteers infected with rhinovirus delayed the onset of symptoms of the disease, but not the infection (Hayden et al., 1988). Finally, anti-idiotypic antibodies directed against virions or viral receptor antibodies also inhibit viral infection (Lentz, 1990).

Another approach to blocking poliovirus or rhinovirus infections uses soluble receptors, i.e. moieties of receptor molecules devoid of the carboxy terminus responsible for anchoring the molecule to the membrane (Kaplan et al., 1990a, b; Marlin et al., 1990; Hoover-Litty and Greve, 1993). Incubation of virus particles with their receptors or truncated (soluble) receptors leads to the formation of complexes and the selective inhibition of infection (Kaplan et al., 1990a, b; Marlin et al., 1990; Hoover-Litty and Greve, 1993). Chimeric molecules of ICAM-1 and immunoglobulins are more effective in inhibiting rhinovirus binding to cells (Martin et al., 1993). The utility of this approach for attacking viral infections in the organism is limited since, for example, mutant viruses that are resistant to neutralization by the receptor readily arise (Kaplan et al., 1990b).

3.3. **Inhibitors of Endosome Function**

3.3.1. **Amines and Ionophores**

Amines are thought to accumulate in endosomes and other organelles of the vesicular system, where they become protonated. Accordingly, they permeate less readily and, thus, cause a rise in the endosomal pH (Fletcher et al., 1965; Seglen, 1983). In contrast, monensin and nigericin (Fig. 13) are ionophores that exchange Na⁺ or K⁺/H⁺ in favour of an H⁺ gradient, thus dissipating the pH gradient created by the proton-ATPase pump (Seglen, 1983). As indicated above, there are, in principle, two possible mechanisms of poliovirus entry, i.e. direct entry through the plasma membrane (Dunnebacke et al., 1969) or internalization in endosomes (Zeichhardt et al., 1985). Poliovirus might use both of these mechanisms in different cell types or even in the same kind of cell. Once the viral particles are in endosomes, they must exit from this organelle in order to initiate translation of the genomic RNA in the cytoplasm. It is still a matter of debate as to whether
poliovirus requires acidification of endosomes to allow the passage of the genome through the endosomal membrane (Madshus et al., 1984b, c; Zeichhardt et al., 1985), or if poliovirus accomplishes this task in a pH-independent fashion (Gromeier and Wetz, 1990; Pérez and Carrasco, 1993). Indirect experiments involving complexing of poliovirus particles with neutral red prior to incubation for several minutes with weak amines or monensin, followed by very long incubation (18–40 hr), have led to the suggestion that poliovirus requires a low-pH step for infectivity (Madshus et al., 1984b, c). Virus entry was evaluated by measuring the cytopathic effect caused by poliovirus complexed with neutral red after several rounds of viral replication (Madshus et al., 1984b, c). High concentrations of amines have also been used, e.g. 0.3 mM chloroquine, or 70 mM NH₄Cl (Zeichhardt et al., 1985). Poliovirus polymerase production was strongly inhibited, even when the compounds were added 1 or 2 hr after virus entry (Zeichhardt et al., 1985). Certainly, the lysosomotropic agents employed have a number of side effects that influence a variety of enzymes and functions apart from raising the endosomal pH (Seglen, 1983; Hiebsch et al., 1991). Hence, it is not surprising that during the long incubation times, where several rounds of viral replication take place, there was inhibition of the virus (Madshus et al., 1984b, c). Shorter incubation times testing the inhibition of poliovirus by chloroquine or monensin during the uncoating period lead to the idea that poliovirus entry is pH-independent (Gromeier and Wetz, 1990; Pérez and Carrasco, 1993). The presence of lysosomotropic agents, such as chloroquine, amantadine, dansylcadaverine or monensin, during poliovirus entry did not inhibit infection (Pérez and Carrasco, 1993).

Studies on the requirement of a low-pH step for the entry of other members of the Picornaviridae family indicate that replication of FMDV is inhibited by lysosomotropic compounds (Carrillo et al., 1984, 1985; Baxt, 1987). A direct action of these agents in the initial steps of virus infection is still uncertain; however, chloroquine was still active in the inhibition of FMDV replication, even when added 2.5 hr after infection (Baxt, 1987), whereas monensin (Neubauer et al., 1987), as well as BFLA1 (Pérez and Carrasco, 1993), blocked the internalization of labelled rhinovirus particles into HeLa cells. It has been claimed that monensin might even increase infection of cells by EMC virus when low-pH medium is used during virus penetration (Madshus et al., 1984a). Entry of EMC virus into HeLa cells is not inhibited by increasing the pH in endosomes (Pérez and Carrasco, 1993).

![Fig. 13. Chemical structures of different compounds: bafilomycin A1, monensin, chloroquine and nigericin.](image-url)
Table 1. Characteristics of Some Toxins that Specifically Enter Together with Virus Particles

| Toxin          | Producer organism       | MW AA                  | Mode of action                      |
|----------------|-------------------------|------------------------|-------------------------------------|
| Diphtheria toxin | Corynebacterium diphtheriae | 58,000 (535 AA)        | ADP-ribosylation/elongation factor EF-2 |
| Exotoxin A     | Pseudomonas aeruginosa  | 54,000 (613 AA)        | ADP-ribosylation/elongation factor EF-2 |
| Ricin          | Ricinus communis        | (A + B chains) 64,000  | Adenine removal from 28S ribosomal RNA |
| (262 AA + 240 AA) |                        |                        |                                     |
| Abrin          | Abrus precatorius       | (A + B chains) 65,000  | Adenine removal from 28S ribosomal RNA |
| (251 AA + 267 AA) |                        |                        |                                     |
| PAP            | Phytolacca americana   | 31,000                | Adenine removal from 28S ribosomal RNA |
| Alpha-sarcin   | Aspergillus giganteus   | 16,000 (150 AA)        | 28S ribosomal RNA cleavage          |
| Mitogillin     | Aspergillus restrictus  | 16,900 (149 AA)        | 28S ribosomal RNA cleavage          |
| Restrictocin   | Aspergillus restrictus  | 16,000 (149 AA)        | 28S ribosomal RNA cleavage          |

The producer organism, the molecular mass (MW), number of amino acids (AA) and the mode of action are indicated.

3.3.2. Bafilomycin A1

Three major classes of proton-ATPases exist in mammalian cells: (1) the mitochondrial F-class ATPases, (2) the plasma membrane proton ATPases of the P-class and (3) the V-class of vacuolar proton-ATPases (Schneider, 1987; Nelson and Taiz, 1989). Each class is distinguished by several parameters, including their sensitivity to inhibitors (Nelson and Taiz, 1989). Endosomes, along with other organelles of the vesicular system, become acidified by the action of the vacuolar proton-ATPases (Schneider, 1987; Nelson and Taiz, 1989). These enzymes pump protons into the organelles at the expense of ATP hydrolysis (Schneider, 1987; Nelson and Taiz, 1989). The two major classes of inhibitors, hitherto used to assess the requirement for a low-pH step during virus infection, are either weak amines (NH4Cl, chloroquine, amantadine, etc.) that raise the endosomal pH by their accumulation of ionophores (monensin, nigericin) that dissipate the proton gradient (Seglen, 1983) (see Section 3.3.1). In some studies, the inhibitor dicyclohexylcarbodiimide (DCCD), which blocks all types of proton-ATPases, has been assayed (Seglen, 1983; Hiebsch et al., 1991). BFLA1 (Fig. 13), a recently discovered antibiotic, is a powerful and selective inhibitor of the vacuolar proton-ATPases (Bowman et al., 1988; Nelson and Taiz, 1989; Zeuzem et al., 1992; Dröse et al., 1993). BFLA1, in contrast to DCCD, which nonspecifically blocks all types of proton-ATPases (Nelson and Taiz, 1989), is a unique compound for assaying the roles that acidification of endosomes and the action of the vacuolar proton-ATPase play in the entry of animal viruses. Viruses such as SFV or VSV, which enter cells through endosomes and need their acidification, are potently inhibited by BFLA1, whereas poliovirus infection is not affected by the antibiotic (Pérez and Carrasco, 1993). Therefore, the entry of poliovirus is not restricted by the three different techniques used to increase the endosomal pH, i.e. (1) the blockade of the vacuolar proton-ATPase with BFLA1, (2) the raising of the endosomal pH by weak amines and (3) the dissipation of the pH gradient with monensin. The effect of BFLA1 on other members of the Picornaviridae family indicates that entry of EMC virus into HeLa cells was not affected by this macrolide antibiotic, whereas rhinovirus was sensitive to its action (Pérez and Carrasco, 1993). The co-entry of toxins, such as α-sarcin, together with poliovirus particles is potently inhibited by BFLA1, indicating that an active vacuolar proton-ATPase is necessary for the early membrane permeabilization induced by poliovirus (see Section 2.4).

3.4. Toxins that Enter Cells Together with Virus Particles

A number of protein toxins are effective inhibitors of protein synthesis (Vázquez, 1979; Jimenez and Vázquez, 1985) (Table 1). The mode of action of these toxins in intact cells involves two steps, namely attachment of the toxin to cell membrane receptors and entry of the toxin, or its effector moiety, into the cytoplasm where enzymatic inactivation of the components of the protein synthesizing machinery occurs. Some toxins, such as diphtheria or Pseudomonas exotoxin A,
inactivate the elongation factor EF-2 by ADP-ribosylation (Aktories, 1992), whereas ricin, abrin, α-sarcin, mitogillin, etc., inactivate ribosomes by acting on the 28S rRNA (Endo et al., 1987; Endo and Tsurugi, 1987; Hartley et al., 1991; Better et al., 1992; Irvin and Uckun, 1992). Some plant toxins, such as mitogillin, restrictocin or α-sarcin, do not bind to receptors, but are potent inhibitors of translation in cell-free systems or in permeabilized cells (Jimenez and Vázquez, 1985; Carrasco and Vázquez, 1983).

Poliovirus is able to co-internalize, and, thus, deliver, protein toxins efficiently into cells (Carrasco, 1981; Carrasco et al., 1989; Lee et al., 1990; Almela et al., 1991). The antiviral potency of these toxins is high because after the toxin has entered along with the infecting virus, the translation of viral proteins does not occur and the formation of the progeny virus is strongly suppressed (Ussery et al., 1977; Yamaizumi et al., 1979; Fernandez-Puentes and Carrasco, 1980; Carrasco, 1981; Carrasco and Esteban, 1982; Irvin and Uckun, 1992). In addition to protein toxins, low molecular weight antibiotics also enter cells early during infection (Carrasco, 1981; Carrasco and Vázquez, 1983). In general, hydrophilic antibiotics are selective inhibitors of viral translation (Carrasco and Vázquez, 1983), thus blocking viral replication at concentrations that do not affect uninfected cells (Lacal and Carrasco, 1983b) (see also Section 4.3.1). The mode of action of different inhibitors on the entry of poliovirus and the co-entry of toxins is illustrated in Fig. 14.

4. TRANSLATION OF PICORNAVIRUS RNA

Each picornavirus possesses a single species of mRNA that is identical to its genome (Harber and Wimmer, 1993). This long RNA (7.2–8.5 Kb) has a single initiator AUG (Meerovitch and Sonenberg, 1993) except for FMDV, where initiation can take place on two different AUGs that are in phase and direct the synthesis of two different L proteins (Belsham, 1992). The translation
Fig. 15. Representation of the non-coding regions of the poliovirus genome: RLP is also known as the IRES region, responsible for the internal initiation of translation. The pseudoknot present in the 3' region is depicted. For further explanation see text.
compared with translation of cellular mRNAs (Wigle and Smith, 1973). More recent studies have analyzed the effects of exogenously added individual initiation factors to rabbit reticulocyte lysates programmed with capped or uncapped mRNAs. Internal initiation on synthetic bicistronic mRNAs that contain the poliovirus 5'-end in the second cistron is increased by addition of eIF-4B and eIF-4F (Anthony and Merrick, 1991), indicating that eIF-4F participates in the translation of naturally uncapped mRNAs (Anthony and Merrick, 1991; Thomas et al., 1991).

Apart from the initiation factor requirement for efficient translation, a number of cellular proteins with binding affinity for picornavirus RNA have been identified, but the roles that these proteins could play during internal initiation of translation, or as "initiation correction factors", deserve further investigation (Meerovitch and Sonenberg, 1993; Meerovitch et al., 1993). Viral proteins can also bind to specific regions on the 5'-end of mRNA and, perhaps, might affect translation. This is the case for poliovirus protein 3CD that binds to the 5'-end (Andino et al., 1990a), or for protein 2A, which enhances translation of poliovirus RNA by an as yet unidentified mechanism (Hambidge and Sarnow, 1992).

One of the most interesting aspects of the translation of picornavirus RNAs is that, in comparison with cellular mRNAs, they require a higher concentration of monovalent cations for optimal translation (Carrasco and Smith, 1976). In fact, under the ionic conditions required to initiate translation efficiently on picornavirus RNA, the initiation on cellular mRNAs is restricted (Saborio et al., 1974; Carrasco and Smith, 1976; Carrasco et al., 1979). The discrimination between viral and cellular mRNAs shown by monovalent cations in cell-free systems is such that at higher cation concentrations, the resulting effects perfectly mimic the shut-off of host translation observed in intact cells after infection (Carrasco and Smith, 1980). The physiological significance of these requirements is related to the fact that viral protein synthesis takes place mainly in a cytoplasm where monovalent ion concentrations have drastically changed (see review by Carrasco et al., 1989) as a consequence of membrane leakiness produced during picornavirus infection. This observation explains the shut-off of host translation during infection that is observed in some members of the Picornaviridae family (Carrasco, 1977, 1987; Carrasco and Castrillo, 1987).

4.1. Picornavirus Proteases

Picornaviruses encode for the proteases responsible for the processing of the polyprotein to produce mature viral proteins (Krausslich and Wimmer, 1988; Hellen et al., 1989; Lawson and Semler, 1990; Palmenberg, 1990). The picornavirus proteases have been classified as cysteine peptidases (Rawlings and Barrett, 1993). The two poliovirus proteases, proteins 2A and 3C, are active in the precursor form and are able to cleave substrates in cis. Indeed, after their proteolytic modification, they can recognize their substrates in trans even in the nascent polypeptide chain. Viral proteases are highly specific and cannot be interchanged between different picornavirus species (Krausslich and Wimmer, 1988; Hellen et al., 1989; Lawson and Semler, 1990; Palmenberg, 1990). Apart from cleaving the viral polyprotein and the polypeptide precursors, they can also cleave a limited number of cellular proteins (Grigera and Tisminetzky, 1984; Urzáinqui and Carrasco, 1989; Falk et al., 1990; Clark et al., 1991; Joachims and Etchison, 1992). The best illustrated example is the cleavage of polypeptide p220 that forms part of the translation initiation factor eIF-4F induced by poliovirus protease 2Apro (Etchison et al., 1982; Lloyd et al., 1988). Cleavage of p220 is not directly accomplished by 2Apro, but by another cellular protease that is activated by 2Apro (Wyckoff et al., 1990, 1992). It was suggested that cleavage of p220 was a crucial step in the inhibition of host translation by poliovirus infection (Etchison et al., 1982). However, recent evidence indicates that this may not be so. Thus, there is no correlation in time between p220 cleavage and the shut-off of host translation (Etchison et al., 1982; Bonneau and Sonenberg, 1987; Pérez and Carrasco, 1992); the presence of some inhibitors of poliovirus RNA synthesis allows complete cleavage of p220, while for hours there is still a substantial amount of host protein synthesis (Bonneau and Sonenberg, 1987; Pérez and Carrasco, 1992); translation of several capped viral mRNAs can take place in poliovirus-infected cells (Alonso and Carrasco, 1982b; Muñoz et al., 1985a; Castrillo and Carrasco, 1987b; Schrader and Westaway, 1990); eIF-4E, an initiation factor that, together with p220 forms eIF-4F, is localized in the nucleus and not in the cytoplasm (Lejbkowicz et al., 1992); monocytic cell lines persistently infected with poliovirus synthesize
proteins even though p220 has been extensively, but not completely, degraded (Lloyd and Bovee, 1993); a poliovirus mutant in 2A that is unable to cleave p220 still shuts down host translation (Bernstein et al., 1985); eIF-4F stimulates the translation of capped or naturally uncapped viral mRNAs (Anthony and Merrick, 1991; Thomas et al., 1991); transient expression of poliovirus protein 2A, the protease that induces p220 cleavage, has a major impact on transcription of a reporter gene, compared with its translation (Davies et al., 1991); finally, disruption of the yeast p220 gene homologue does not block cellular growth (Lanker et al., 1992).

4.2. Proteases as Targets of Antipicornavirus Agents

Precise action of picornavirus proteases is vital for generating the mature viral proteins that are, in turn, required for a number of viral processes, including replication of the picornavirus genome (Krausslich and Wimmer, 1988; Hellen et al., 1989; Lawson and Semler, 1990; Palmenberg, 1990). Thus, it seems feasible that selective interference with protease function would lead to the specific inhibition of viral growth (Korant et al., 1986; Hellen and Wimmer, 1992a). Indeed, a number of agents have been described as inhibitors of protease function (Korant et al., 1986; Korant, 1987, 1990, 1991) and can be broadly classified in two categories: (1) compounds that modify the conformation of the viral polyprotein and render it refractory to protease action and (2) inhibitors of the proteases themselves.

With respect to the first category, a number of amino acid analogues (Jacobson et al., 1970; Jacobson and Baltimore, 1968), zinc ions (Korant et al., 1974) or high temperature lead to conformational changes of the poliovirus polyprotein, which is then no longer recognized by viral proteases (Korant, 1990, 1991). However, this approach is not selective for inhibition of viral proteases since cellular proteins are also affected.

A number of amino acid analogues have been used to inhibit the proteolytic degradation of the picornavirus polyprotein. In fact, these agents were employed to identify picornavirus precursors and to block the formation of mature proteins. Compounds such as DL-ethionine (Brown and Ackermann, 1951), TPCK (tolylsulfonylphenylalanyl chloromethyl ketone), TLCK (tolylsulfonyl lysyl chloromethyl ketone) (Korant, 1972), ZPCK (carbobenzyloxyphenylalanyl chloromethyl ketone) (Summers et al., 1972), ZLCK (carbobenzoxyleucyl chloromethyl ketone) (Korant et al., 1979), canavanine or fluorophenylalanine (Brown et al., 1961; Levintow et al., 1962; Scharff and Levintow, 1963) (Fig. 16) inhibit the proteolytic processing of the picornavirus polyprotein.

4.2.1. Cystatin C

The activities of cysteine proteases are controlled by a set of protein inhibitors that constitute the cystatin superfamily of proteins. Cystatin C is a human cysteine proteinase inhibitor present in extracellular fluids. Cystatins are small proteins (110-120 amino acids) that bind and selectively inhibit cysteine proteases. Human cystatin C, obtained from human serum, and human stefin B, obtained from spleen, also bind and inhibit poliovirus protease 3C and block the replication of poliovirus in cultured cells (Korant et al., 1985, 1986). Not only cystatin C, but also peptides that mimic the proteinase-binding center of cystatin C, such as Z-LVG-CHNHz (N-benzyloxycarbonyl-leucyl-valyl-glycine diazomethylketone), show antiviral activity against poliovirus and herpes simplex virus (see Section 4.2.2) (Bjorck et al., 1990).

4.2.2. Peptidyl Diazomethyl and Chloromethyl Ketones

The use of peptides that mimic the viral cleavage site provides a potential for the development of new protease inhibitors, because they inhibit protease function (Green and Shaw, 1981). Therefore, analogues of the recognition site of viral proteases have a modified C-terminal or the N-terminal blocks viral replication (Bjorck et al., 1990; Korant, 1990). Tri-, tetra- or pentapeptides having, in the C-terminal, chloromethyl ketone or methyl ketone moieties, were active against poliovirus and rhinovirus (Kettner and Korant, 1987).
4.2.3. **E-64 (L-Trans-Epoxysuccinyl-Leucylamido(4-Guanidine) Butane)**

The thiol protease inhibitor E-64 (Fig. 16) is a natural product isolated from *Aspergillus japonicus*. It has also been chemically synthesized. E-64 and leupeptin are inhibitors of the cysteine protease calpain (Mehdi, 1991). E-64 derivatives lacking charged groups inhibit intracellular proteases (Mehdi, 1991). E-64 specifically blocks the L-protease of FMDV, leading to a reduction in virus yields (Kleina and Grubman, 1992). Addition of a permeable analogue to cultured cells infected with FMDV decreases viral RNA synthesis, cleavage of p220 and retardation of the virus-induced shut-off of host translation (Kleina and Grubman, 1992). It is not yet known whether this last effect is due to inhibition of the L-protease itself or to the general retardation of viral replication. E-64 and its permeable derivatives are nontoxic in animals, and doses of 400 mg/kg of body weight are well tolerated by various administration routes (Komatsu *et al.*, 1986; Kleina and Grubman, 1992).

![Chemical structures of different compounds: spiro indolinone β-lactam, ethionine, canavanine, thysanone, ZLCK, TPCK and E-64.](image)
Fig. 17. Modification of membrane permeability and phospholipase action late after poliovirus infection. The modification of the membrane late during viral infection leads to a disbalance of the ionic gradients and the increased entry of unpermeant inhibitors. The activation of phospholipase C results in the release of IP3, choline and phosphorylcholine from phospholipids.

4.2.4. Spiro Indolinone β-Lactams and Thysanone

A series of spiro indolinone β-lactams have been synthesized and their activity assayed against poliovirus and rhinovirus 3C proteases (Skiles and McNeil, 1990). The sulfonamide derivative shown in Fig. 16 was active not only against these picornavirus proteases, but against other cellular proteases such as human leukocyte elastase and cathepsin G (Skiles and McNeil, 1990).

Thysanone (Fig. 16) is a novel naphthoquinone isolated from the fungus Thysanophora penicilloides, which is active against rhinovirus 3C protease (Singh et al., 1991). The selectivity of these compounds in cell culture has yet to be investigated.

Although viral proteases have repeatedly been suggested as potentially effective targets for the inhibition of viral growth (Korant, 1987; Hellen and Wimmer, 1992a), no clinical results have been reported to support this premise.

4.3. Modification of Membrane Permeability by Picornaviruses

The most drastic modifications of membrane permeability occur in the late phase of the replication cycle of many cytolytic viruses, and a detailed review on this subject has already been published (Carrasco et al., 1989) (Fig. 17). The major salient feature is that a viral gene product is required for the development of late modifications in membrane permeability. This modification starts in HeLa cells at about the third hour after infection with poliovirus (Nair et al., 1979; Lacal and Carrasco, 1982; Muñoz and Carrasco, 1983; Nair, 1984; López-Rivas et al., 1987; Garry, 1989), or at the second hour p.i. in cells infected with SFV (Garry et al., 1979; Ulug et al., 1987). Thus, the majority of viral macromolecules are synthesized in cells with an altered plasma membrane in a cytoplasm where the concentrations of ions, and perhaps pH and divalent cations, are gradually changing (Carrasco and Smith, 1976; Muñoz et al., 1985a, b; López-Rivas et al., 1987; Holsey et al., 1990). The mechanism of membrane permeabilization by poliovirus is fairly well understood. There is an overall increase in permeability to a variety of molecules (Carrasco et al., 1989, 1993), and the modification of the membrane is such that the gradients of monovalent cations are destroyed. In addition, other metabolites and compounds that are excluded by the membrane leak out from cells and can readily pass to the cytoplasm (Egberts et al., 1977; Benedetto et al., 1980; Nair, 1981).

An updated view of the modifications of membrane permeability by animal viruses is shown in
Fig. 17. The putative viral protein responsible for membrane modification associates with membranes once it is synthesized and travels through the vesicular system to the plasma membrane. As we have already indicated, these viral proteins may possess an ionophore-like activity (Carrasco, 1977, 1987; Carrasco et al., 1993), such that the pH and ionic content of vesicles of the vesicular system will be modified. Once these proteins reach the plasma membrane, they increase the permeability to ions, leading to the disruption of the membrane potential (Lacal and Carrasco, 1982, 1983a). We have suggested that these changes of ions and pH in the cytoplasm influence an array of cellular functions, including the translation of host mRNAs (Carrasco, 1977, 1987; Carrasco et al., 1993).

With respect to the mechanism of late membrane permeabilization, we have found that phospholipase A2 activity is inhibited early following poliovirus infection, whereas the phospholipase C that hydrolyses phosphatidylinositol is stimulated from the third hour p.i. (Guinea et al., 1989). Thus, in poliovirus-infected cells, the amount of inositol triphosphate (IP3) present in the cytoplasm constantly increases (Guinea et al., 1989). This increase would lead to an augmentation of free calcium. However, we are not aware of any analysis of free calcium levels in cells infected by picornaviruses. Recently, we have analyzed in detail the activation of the phospholipase C that hydrolyses phosphatidylcholine, and found that both choline and phosphorylcholine drastically increase in the medium of poliovirus-infected cells from the third hour of infection (Irurzun et al., 1993).

It is clear that a correlation in time exists between the increase in membrane permeability and the activation of phospholipase C in poliovirus-infected cells (Guinea et al., 1989; Irurzun et al., 1993). However, we do not know yet if there is a cause-effect relationship between them. A number of protein toxins, such as melittin, that modify membrane permeability also activate phospholipase activity (Durkin and Shier, 1981; Pellkofer et al., 1982). It is tempting to speculate that poliovirus encodes a protein responsible for modifying the membrane (Lama and Carrasco, 1992a, b; Lama et al., 1992). This protein could be poliovirus protein 3A, a membrane protein that contains an amphipathic helix. A similar motif is found in other viral membrane proteins that modify membrane permeability (Pinto et al., 1992; Carrasco et al., 1993). Such viral proteins that are responsible for increasing membrane permeability have been collectively referred to as viroporins (Carrasco et al., 1993). The accumulation of a sufficient number of copies of such a viroporin (Carrasco et al., 1993) would form pores in the membrane that may lead per se to the permeabilization of the membrane. A secondary effect could be the activation of phospholipase C, to generate diacylglycerol, which, in turn, also increases membrane permeability (Felix, 1982; Otero and Carrasco, 1988).

4.3.1. Nonpermeant Translation Inhibitors

In principle, translation inhibitors that have their target on components of the protein synthesizing machinery would block viral and cellular translation nonspecifically. The action of compounds such as puromycin (Levintow et al., 1962) or pactamycin (Summers and Maizel, 1971; Taber et al., 1971) have been used to study the dependence of viral RNA synthesis on viral translation (Levintow et al., 1962) or to map the viral proteins on the picornavirus genome (Lawson and Semler, 1990). However, there is no selectivity in this inhibition compared with the blockade of host protein synthesis in uninfected cells. A selective way to block protein synthesis in animal virus-infected cells was devised based on modification of membrane permeability during virus infection. This approach employs the use of hydrophilic translation inhibitors that do not easily cross the membrane barrier of normal uninfected cells, but readily enter into a number of animal virus-infected cells (Carrasco, 1978; Contreras and Carrasco, 1979; Vázquez, 1979; Carrasco et al., 1989). These studies showed that the bulk of picornavirus protein synthesis occurs in cells where the membrane has been drastically modified (Carrasco et al., 1989). The presence of these translation inhibitors not only blocked selectively the synthesis of viral proteins, but also blocked the formation of new infectious virus, indicating that virus assembly takes place largely after the membrane has been modified (Benedetto et al., 1980). Hence, inhibitors of translation that are hydrophilic and do not inhibit protein synthesis efficiently in intact cells are able to selectively block translation in a variety of animal virus-infected cells (Carrasco, 1978; Contreras and Carrasco,
Fig. 18. Chemical structures of different compounds: gougerotin, blasticidin S and anthelmycin.

Fig. 19. Chemical structures of different compounds: GppCH₂P, hygromycin B and edeine A1.
used as a test to measure changes in membrane permeability not only in cells infected with animal viruses, but also in any cell type (eukaryotic or prokaryotic) where membrane permeability has been altered (Alonso and Carrasco, 1981a, b, 1982a; Otero and Carrasco, 1987a; Lama and Carrasco, 1992b).

The formation of infectious virus by these agents is strongly diminished at drug concentrations that do not affect translation in uninfected cells (Benedetto et al., 1980; Lacal et al., 1980; Lacal and Carrasco, 1983b). Therefore, cell killing by low multiplicities of poliovirus is prevented by treatment of cell monolayers with hygromycin B (Lacal and Carrasco, 1983b). This selectivity is not achieved by other inhibitors of translation that enter equally well in virus-infected or uninfected cells (Contreras and Carrasco, 1979; Lacal and Carrasco, 1983b). This approach has not been exploited in animals because some of the nonpermeant antibiotics that block translation are not very potent, as occurs with guanylyl methylene diphosphate (Dawson et al., 1979), or showed toxicity (unpublished results), perhaps due to different cellular susceptibilities in the animal after prolonged treatment. However, the approach of using inhibitors of any viral or cellular function that selectively enter into virus-infected cells early or late during infection, as a result of modification of membrane permeability, remains open. In other words, selectivity can be conferred on antiviral compounds by adding charged residues to make them less permeable in uninfected cells, whereas these charged molecules will readily enter virus-infected cells. In fact, we have observed that highly charged compounds, such as sulphated polysaccharides, suramin or the dye trypan blue, show good antiviral activity (Alarcón et al., 1984c), and the entry of sulphated polysaccharides into cells is enhanced by poliovirus particles (González and Carrasco, 1987) (see Section 2.4). These agents have been used by a number of laboratories in tests for antiviral effects against several animal viruses, including human immunodeficiency virus (HIV) (Mitsuya et al., 1984, 1988; Balzarini et al., 1986; González et al., 1987; Zuckerman, 1987; Schols et al., 1990; Callahan et al., 1991; McClure et al., 1991).

5. PICORNAVIRUS GENOME REPLICATION

Once the viral genome is liberated in the cytoplasm after poliovirus entry, it is recognized by the ribosomes and the translation machinery as mRNA (Sonenberg, 1990). Translation of the input viral RNA gives rise to a number of virus-encoded proteins that are necessary to replicate the genome (Wimmer et al., 1987; Semler et al., 1988; Richards and Ehrenfeld, 1990). Although many details of the steps involved in the replication of poliovirus RNA have been unravelled, this is perhaps one of the least understood processes in molecular terms. This is particularly so for the initiation of new RNA chains, the possible role played by some cellular proteins and the requirement of membranes in genome replication (Wimmer et al., 1987; Semler et al., 1988; Richards and Ehrenfeld, 1990). The replication of an RNA involving only RNA, and not DNA, intermediates, as occurs with retroviruses or hepadnaviruses, is perhaps one of the most primitive processes in the multiplication of biological molecules. An understanding of how picornaviruses accomplish their replication can shed light on the essential requirements of one of the most primitive forms of life and elucidate how other more complex RNA-containing viruses are able to replicate their genomes in infected cells. In addition, once the requirements for the replication of an RNA genome have been defined, they can be used for the amplification of any kind of RNA molecule, either in intact cells or in cell-free systems, as already achieved with phage RNA (Wu et al., 1992).

One of the essential steps required for the amplification of a positive-stranded RNA molecule is the copying of this RNA to its complementary chain that, in turn, will be used as a template for synthesis of more positive-strand RNA molecules (Wimmer et al., 1987; Semler et al., 1988; Richards and Ehrenfeld, 1990) (Fig. 20). Thus, in picornavirus-infected cells, the only two classes of RNA molecules present are: the genomic positive-strand RNA or an exact copy of this RNA, the negative-strand RNA (Wimmer et al., 1987; Semler et al., 1988; Richards and Ehrenfeld, 1990). There are approximately 100 times more (+)RNA than (−)RNA molecules in the infected cells (Hewlett et al., 1977). This relation varies, depending upon the cell line infected (Lopez Guerrero et al., 1991). The (−)RNA is found associated with (+)RNA, either as a replicative form (RF)
Fig. 20. Schematic representation of picornavirus RNA replication. The polymerase (protein 3D) elongates both (+) and (−)RNA. The terminal protein 3B (VPg) is covalently bound to the 5'-end of any kind of viral RNA (ssRNA, single-stranded RNA).

of double-stranded RNA (ds-RNA) or as a replicative intermediate (RI) of partially double-stranded and partially single-stranded RNA (Baltimore, 1966; Baltimore and Girard, 1966; Hewlett et al., 1977). The single-stranded RNA found in cells as such is of positive polarity and it can be encapsidated in virions or serve as mRNA for the synthesis of viral proteins. In the encapsidated form, the virion RNA has a covalently bound protein (VPg) at the 5'-end of the molecule (Wu et al., 1978). The RI found in cells consists of a complete (−)RNA molecule that is used as template to make copies of (+)RNA. These are present as nascent RNA molecules (4–8 nascent (+)RNA chains per RI), all of which contain a VPg molecule bound to the 5'-end. In fact, this molecule is present in all forms of RNA, positive or negative, that form part of RI or RF (Wimmer et al., 1987).

It is well established that the enzyme required to elongate both (−) or (+)RNA is 3Dpol (Flanegan and Baltimore, 1977; Flanegan and Van Dyke, 1979). This enzyme, however, is unable to initiate the synthesis of new RNA chains (Wimmer et al., 1987; Semler et al., 1988; Richards and Ehrenfeld, 1990). 3Dpol is a polymerase that is able to copy RNA, using a primer and another RNA molecule as template (Richards and Ehrenfeld, 1990). In fact, the first enzyme of this kind found in mammalian cells was described by Baltimore et al. (1963) in picornavirus-infected cells.
Apart from $3D^{pol}$, the total number of proteins required for replication of the picornavirus genome has not yet been established. Virus-encoded proteins are necessary and cellular proteins may also be involved (Wimmer et al., 1987; Semler et al., 1988; Richards and Ehrenfeld, 1990). In addition, viral (or cellular) proteins need to be synthesized continuously for participation in the synthesis of viral RNAs, because inhibition of protein synthesis in picornavirus-infected cells leads to almost immediate arrest of viral RNA synthesis (Fig. 21).

Of the viral proteins implicated in the replication of poliovirus genomes, certainly $3D^{pol}$ participates in the elongation process and VPg is also necessary (Wimmer et al., 1987; Semler et al., 1988; Richards and Ehrenfeld, 1990). It remains unclear if VPg acts as a primer to initiate the synthesis of new RNA chains or if VPg is added later after initiation has been accomplished (Takegami et al., 1983; Tobin et al., 1989), and these two alternative possibilities are illustrated in Fig. 22. According to model A, VPg, either as such or as the precursor 3AB, will, in the presence of UTP and a still unidentified protein, be uridylated to VPg-UMP and then further elongated to VPg-pUpUp (Takeda et al., 1986). This substrate will be used as a primer to elongate the RNA chain (Takeda et al., 1986). Both $(-)$ and $(+)$RNA start with pUpUp at the 5'-end, and it is possible that VPg alone does not participate as such in the formation process, but as a precursor molecule 3AB (Wimmer et al., 1987). Thus, 3A would serve to anchor 3AB to the membrane in such a way that 3B would be "hanging" from the membrane and ready to be uridylated (Takegami et al., 1983; Wimmer et al., 1987). Once 3AB-Up-Up is synthesized, or after the elongation process has commenced, $3C^{pol}$ will hydrolyze 3AB, releasing 3B from the membrane, while 3A would remain imbedded in the lipid bilayer (Wimmer et al., 1987). We have indicated that 3A is a firm candidate to modify membrane permeability and to lyse the infected cells (Lama and Carrasco, 1992a; Carrasco et al., 1993). In model B of Fig. 22, 3B (VPg) does not participate in a real initiation event, since the 3'-OH group present in the hairpin can be used directly by $3D^{pol}$ to elongate the RNA chain. 3B (VPg) will bind somewhere during elongation in a reaction catalyzed by 3B itself with no requirement for nucleotide hydrolysis (Tobin et al., 1989). Thus far, this model has not been confirmed by other laboratories.

Apart from the roles that $3D^{pol}$, 3B (VPg), 3A and, indirectly, $3C^{pol}$ play in genome replication, other viral proteins are also implicated in this process although their functions are more obscure (Wimmer et al., 1987; Semler et al., 1988; Richards and Ehrenfeld, 1990). Genetic studies indicate that the functions of 2C, 2B and perhaps $2A^{pol}$ are also necessary for replication of viral RNA.

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**Fig. 21.** Effect of cycloheximide on viral RNA synthesis in poliovirus-infected HeLa cells. Cycloheximide (50 $\mu$M) was added at the times indicated by the arrows and RNA synthesis was measured during 1-hr intervals in the presence of actinomycin D (Almela and Carrasco, unpublished observations).
Fig. 22. Two alternative models for the initiation of poliovirus RNA synthesis. Model A: initiation occurs by priming with VPg. Model B: VPg does not participate in a real initiation step and is bound to the 5'-end of viral RNA by an autocatalytic event. For more details see text (ssRNA, single-stranded RNA).
in 3Dpol (Cho et al., 1993). In fact, we have suggested that 2C may represent a new class of small GTP-binding proteins and its role could be to direct the trafficking of RNA replicative complexes through the vesicular system (Rodriguez and Carrasco, 1993). Clearly, more experiments are needed to clarify the activity of 2C in the replication of the poliovirus genomes.

Protein 2B, either as such or as the precursor 2BC, is also required to replicate the picornavirus genome (Johnson and Sarnow, 1991). Poliovirus with mutations in 2B is impaired in its ability to synthesize viral RNA, but there is no clue as to the exact role that this protein has in this process (Bernstein et al., 1986; Sarnow et al., 1990; Kirkegaard, 1992). Mutations in protein 2B cannot be complemented in trans, suggesting that only the actual 2B proteins made on a particular RNA are functional in the replication of that RNA (Johnson and Sarnow, 1991). Indeed, this phenomenon very much complicates the possibility of using the picornavirus system as a model for the replication of any kind of RNA in cells or in cell-free systems (Kirkegaard, 1992). Finally, 2Apro may also participate in RNA replication. Apart from cleaving itself from P1, while the nascent polypeptide chain is being synthesized (Krausslich and Wimmer, 1988), 2Apro must have another role in viral growth. This idea is strongly supported by the fact that a poliovirus monster recently constructed, containing the P1 region separated from P2/P3 by the EMC 5' untranslated region, requires the presence of 2Apro, even though P2 proteins are now initiated on an internal AUG (Molla et al., 1992).

5.1. Cellular Proteins Involved in Picornavirus Genome Replication

The major evidence that host-factors are required for the replication of the picornavirus genome comes from cell-free systems (Semler et al., 1988; Richards and Ehrenfeld, 1990). These systems are extremely useful for clarifying this process in molecular detail, but they are also prone to artifacts. Thus, a 67 kDa protein with uridylation activity has been isolated from infected cells that can replace the oligo(U) primer in the initiation of new poliovirus RNA chains (Dasgupta et al., 1980; Baron and Baltimore, 1982; Dasgupta, 1983). This host factor was identified as the protein kinase that phosphorylates elf-2 (Morrow et al., 1985). The role that such an activity may have in poliovirus-RNA replication has not been elucidated (Wimmer et al., 1987; Semler et al., 1988; Richards and Ehrenfeld, 1990), and the requirement of host factors for picornavirus RNA replication also requires further investigation.

5.2. Cellular Membranes and Picornavirus RNA Synthesis

The replication of poliovirus genomes takes place in close association with newly made membrane vesicles (Wimmer et al., 1987; Semler et al., 1988; Richards and Ehrenfeld, 1990). During poliovirus infection, the synthesis of membranes is profoundly modified, such that the cytoplasm is loaded with vesicles of various sizes that form a distinct perinuclear structure (Dales et al., 1965; Caliguiri and Tamm, 1970a, b; Mosser et al., 1972a). This distinctive structure was termed viroplasm (Dales et al., 1965). The synthesis of phospholipids increases after poliovirus infection to provide components for membrane generation (Mosser et al., 1972b). The poliovirus replicative complexes are tightly associated with these newly made membranes (Girard and Baltimore, 1967; Caliguiri and Tamm, 1969, 1970b; Bienz et al., 1990, 1992; Troxler et al., 1992), although little is known about the origin of the membranous vesicles that appear in poliovirus-infected cells or the connection between these vesicles and other membrane compartments. The in vitro synthesis of poliovirus minus- or plus-stranded RNA has only been achieved using a crude membranous replication complex (Girard, 1969; Etchison and Ehrenfeld, 1981; Takeda et al., 1986). Prevention of lipid synthesis results in an abrupt inhibition of viral RNA synthesis (Guinea and Carrasco, 1990, 1991), suggesting that the replication of viral genomes requires continuous membrane formation (see Sections 6.10 and 6.12).

Elucidating the mechanisms used by poliovirus to replicate its genome is of interest for a number of reasons. For example, an understanding of the steps and proteins involved in this process sheds light on the relationship between the virus and the host cell (Koch and Koch, 1985; Wimmer et al., 1987; Semler et al., 1988; Richards and Ehrenfeld, 1990). The complete synthesis of poliovirus in cell-free systems has already been achieved (Molla et al., 1991; Barton and Flanegan, 1993), and
this system should help to clarify the steps and components involved in poliovirus RNA replication. In addition, there is an interplay between the knowledge gained on poliovirus RNA synthesis and the inhibitors of this process, since a detailed understanding of the steps followed by poliovirus and other animal viruses in replicating their genomes is important for exploiting new approaches that interfere with the replication of these pathogens. Moreover, a better understanding of the mode of action of inhibitors of poliovirus RNA replication may help to elucidate this process.

6. COMPOUNDS THAT BLOCK PICORNAVIRUS RNA SYNTHESIS

6.1. Guanidine

Guanidine (Fig. 23) is a basic compound, positively charged at neutral pH, and is a natural constituent of animal serum. It is active against some members of the Picornaviridae and Togaviridae families (Friedman, 1970) and is, in many ways, a model inhibitor of poliovirus, despite the fact that it is one of the least potent agents against viral RNA synthesis (0.2–3 mM) (Tershak, 1974, 1982). Several guanidine derivatives have nevertheless been synthesized with more potent antiviral activity (Bucknall et al., 1973). Soon after the discovery of guanidine as an antiviral agent (Rightsel et al., 1961), poliovirus mutants resistant to or dependent on guanidine were described (Loddo et al., 1963; Nakano et al., 1963). The picornaviruses inhibited by guanidine are poliovirus, rhinovirus and FMDV, whereas EMC virus is resistant to the compound (Caliguiri and Tamm, 1973; Tershak et al., 1982). Guanidine has no adverse effects on cell growth at concentrations that block virus growth and has no direct effect on isolated virions, nor on virus attachment. When the inhibitor is added 3 hr after infection, it does not block translation, whereas the incorporation of [3H]uridine into poliovirus RNA is suppressed. If the compound is added after virus entry, the synthesis of viral proteins and of RNA are both prevented (Caliguiri and Tamm, 1973; Tershak et al., 1982). Moreover, under low or moderate multiplicities of infection, guanidine largely prevents the shut-off of host protein synthesis induced by poliovirus, whereas at high multiplicity of infection, the kinetics of the early shut-off are similar in the absence or in the presence of inhibitory concentrations of guanidine (Lacal and Carrasco, 1983a). For details on the initial studies on guanidine, the reader is referred to comprehensive reviews by Caliguiri and Tamm (1973) and Tershak et al. (1982).

Early studies on the mode of action of guanidine indicated that this compound blocked the

![Structure of guanidine and poliovirus protein 2C](image)

Fig. 23. Structure of guanidine and poliovirus protein 2C. The sequences on 2C containing the GKS motif and the region controlling guanidine-resistant or -dependent mutants are shown.
initiation of new rounds of viral RNA synthesis, but allowed completion of already initiated RNA strands (Caliguiri and Tamm, 1968a, b, 1973; Tershak et al., 1972). It is not known with certainty whether guanidine is a selective inhibitor of the synthesis of plus-strand RNA, or blocks equally well the synthesis of plus- or minus-strand RNA. This indecision is due to the fact that it is difficult to block the synthesis of one strand of RNA without affecting the other. Careful kinetic analyses, using various concentrations of guanidine added at different times after infection, have not, to our knowledge, been carried out. Recently, the complete synthesis of infectious poliovirus has been accomplished in a cell-free system. In this system, poliovirus genomic RNA is added to a HeLa cell extract and after several hours of incubation, infectious poliovirus particles are made (Molla et al., 1991). Guanidine blocks the formation of these particles, indicating that at least under some conditions, the synthesis of plus-strand RNA and the initiation of RNA takes place \textit{in vitro} (Molla et al., 1991).

The inhibition by guanidine of poliovirus or FMDV can be reversed by several amino acids, such as methionine, valine, leucine or threonine (Dinter and Bengston, 1964; Lwoff, 1965), and by other agents, including methylpropanolamine, ethanolamine or choline (Loddo et al., 1966; Philipson et al., 1966). This reversal very much depends on the host cell type analyzed. Transport of guanidine into HeLa cells involves at least two possible mechanisms (Nair, 1987b) that are regulated by the concentrations of cations in the medium (Nair, 1987a). Some antiguanidine agents can block this transport, but this may not constitute the basis of their antiguanidine action; rather they could antagonize guanidine intracellularly by an unknown mechanism (Nair, 1987b).

As indicated earlier in this section, the feasibility of generating mutants allowed genetic mapping, which provided information on the viral protein that was the target of guanidine action (Anderson Sillman et al., 1984; Pincus et al., 1987). Initial studies suggested that capsid proteins, proteases or even VPg were the targets of guanidine. Very detailed analyses of both guanidine-resistant and guanidine-dependent mutants of poliovirus indicate that they map in protein 2C (Pincus and Wimmer, 1986; Pincus et al., 1986; Baltera and Tershak, 1989), and similar conclusions were reached for FMDV guanidine mutants (Saunders and King, 1982). Analyses of the polypeptides of FMDV or poliovirus guanidine-resistant mutants suggested that protein 2C was responsible for this trait. Transfection of COS cells, with plasmids containing cDNA fragments corresponding to poliovirus protein 2C from guanidine-resistant or guanidine-dependent mutants, and with plasmids containing infectious clones of wild-type poliovirus, gave rise to both types of mutant virus (Pincus and Wimmer, 1986). Sequence analyses indicated that mutations in amino acid residue 179 of protein 2C conferred resistance to high guanidine concentrations, whereas changes in residue 187, combined with mutations at position 142, 225 or 227, generate guanidine-dependence (Pincus et al., 1987). Modifications in residue 164 or 187 of poliovirus protein 2C, combined with mutations at residues 223 or 295 and 309, could also confer guanidine resistance (Baltera and Tershak, 1989). Finally, the frequency of reversion of guanidine-dependent mutants of poliovirus to the resistance phenotype has established a mutation frequency of about $6 \times 10^{-4}$, a number consistent with the genetic variability of RNA-containing viruses (Domingo, 1989, 1992; Holland et al., 1992; Drake, 1993).

An updated view of the mode of action of guanidine could be as follows: guanidine acts on protein 2C, perhaps inhibiting the interaction of this protein with membranes (Bienz et al., 1992). Protein 2C is a GTPase that may be involved in trafficking the viral RNA replication complexes through the vesicular system (Rodriguez and Carrasco, 1993). This traffic is necessary for poliovirus, but not EMC virus, genome replication (Irurzun et al., 1992). Thus, guanidine blocks the activity of 2C, the traffic of replication complexes and, hence, the initiation of viral RNA synthesis. This mode of action may also explain why guanidine affects other steps, such as the assembly of new virions that also takes place in close association with the newly-formed membrane vesicles (Koch and Koch, 1985).

Guanidine and some of its derivatives have been used in several pharmacological studies (Davidoff, 1973). This compound provokes an enhanced nerve excitability after injection into frogs and may play a role in the pathogenesis of uremic poisoning. Although the serum levels of guanidine are similar in control and uremic patients, the excretion of methylguanidine is higher in patients with renal failure (Stein and Micklus, 1973). These findings, together with the fact that high concentrations (~1 mM) of guanidine are required for efficient blockage of poliovirus \textit{in vitro},
Picornavirus inhibitors

indicate that guanidine cannot be used therapeutically. Guanidine is ineffective for the treatment of poliovirus infection in animals, because of the rapid secretion of this compound and the rapid development of guanidine-resistant viruses in animals (Caliguiri and Tamm, 1973). In fact, administration of guanidine alone to mice infected with echovirus 9 or coxsackie virus A9 did not protect them. A partial response was observed after combination of 2-(α-hydroxybenzyl)-benzimidazole (HBB) and guanidine, as indicated by several days’ delay, before death (Eggers, 1976).

6.2. Thiosemicarbazones

Thiosemicarbazones (Fig. 24) have been used as therapeutic agents for the treatment of tuberculosis and as antiviral agents against several animal viruses (Levinson, 1975; Pfau, 1982). Isatin-β-thiosemicarbazone inhibits vaccinia virus and rhinoviruses (Katz and Feliz, 1977; Pennington, 1977), but has no effect against poliovirus (Pearson and Zimmerman, 1969). The activity of methisazone (N-methyl-isatin-β-thiosemicarbazone) against several picornaviruses was reported (Bauer et al., 1970), and various species of viruses, including poliovirus, were directly inactivated upon contact with methisazone in the presence of CuSO₄ (Fox et al., 1977). 1-Methyl-4',4'-dibutyl-isatin-β-thiosemicarbazone (busatin) blocks poliovirus (O'Sullivan and Sadler, 1961; Pearson and Zimmerman, 1969), interfering with the synthesis of viral RNA when added at any time during infection (Pearson and Zimmerman, 1969). In contrast with the action of HBB (see Section 6.3) or guanidine, busatin blocks poliovirus RNA synthesis in cell-free systems (Pearson and Zimmerman, 1969), and in this respect shows similarity to gliotoxin (Rodriguez and Carrasco, 1992).

Some derivatives of thiosemicarbazones, the 3-substituted triazinoindoles (SKF 21687, SKF 30097, SKF 36800, SKF 40491) (Fig. 24), are broad-spectrum antipicornavirus agents that block several rhinovirus strains (Matsumoto et al., 1972; Pfau, 1982). SKF 40491 has antirhinovirus activity when administered intranasally to gibbons, but not when used orally (Pinto et al., 1972). SKF 21687 and SKF 30097 had no effect on rhinovirus infection in humans (Togo et al., 1973b).
6.3. Benzimidazoles

Several benzimidazole derivatives (Fig. 25) show activity against a number of animal viruses (Caliguiri and Tamm, 1973; Tamm and Sehgal, 1978; Eggers, 1982, 1985). For example, HBB is a selective inhibitor of picornavirus replication that does not inactivate virion particles, but blocks the replication of viral RNA without affecting the synthesis of cellular RNA (Eggers and Tamm, 1962; Singh et al., 1963). The structure–activity relationships in HBB derivatives are fairly well documented (Caliguiri and Tamm, 1973; Tamm and Sehgal, 1978; Eggers, 1982, 1985), showing that the benzo group and the hydroxybenzyl residue at position 2 are critical for activity. Some HBB derivatives have been reported to be more active than HBB itself (Caliguiri and Tamm, 1973; Tamm and Sehgal, 1978; Eggers, 1982, 1985).

HBB is not a broad-spectrum antipicornavirus agent, being active against poliovirus and some coxsackie viruses, but devoid of activity against rhinoviruses, FMDV or hepatitis A virus (Siegl and Eggers, 1982). The concentration of HBB required for activity against poliovirus is similar to that of guanidine, whereas HBB is about 10-fold more potent than guanidine against coxsackie virus. The action of HBB on poliovirus, as found for that of guanidine and flavones, is reversible. Upon withdrawal of these compounds, virus growth resumes (Caliguiri and Tamm, 1973; Tamm and Sehgal, 1978; Eggers, 1982, 1985).

Studies on the mode of action of HBB indicate that it has no effect on early steps of infection, such as virus entry and uncoating (Eggers and Tamm, 1962). Addition of the compound after poliovirus entry prevents the appearance of viral proteins, because viral RNA replication is blocked. Thus, the incorporation of labelled uridine into viral RNA and the formation of poliovirus-specific RNA, as analyzed by dot-blot hybridization, are inhibited by HBB (Eggers and Tamm, 1963a; González et al., 1990b). This compound has no effect on in vitro synthesis of viral RNA, and it is still not known if the initiation or elongation steps are selectively blocked by this agent in vivo.

A number of picornavirus mutants that are resistant to or dependent on HBB have been isolated, but they are not cross-resistant to guanidine (Eggers and Tamm, 1963b). The dependent mutants require HBB for the replication of viral RNA, and HBB can be replaced by guanidine in this respect (Eggers and Tamm, 1963b). Mutant HBB-dependent viruses back-mutate to the sensitive phenotype with high frequency (\( \sim 10^{-4} \)) (Eggers and Tamm, 1965; Caliguiri and Tamm, 1973), this figure being similar to the mutation rate of picornaviruses for other traits investigated (de la Torre et al., 1990; Domingo, 1992).

![Fig. 25. Chemical structures of different compounds: HBB, enviroxime, 2-benzylbenzimidazole, hydroxyethyl-benzimidazole and hydroxybenzyl-imidazole.](image-url)
Picornavirus inhibitors

HBB and guanidine act synergistically (Singh et al., 1963), suggesting that the exact target inhibited by each compound is different, although they could act on the same viral protein. Investigations of the antiviral effects of HBB in mice and monkeys have been carried out (Caliguiiri and Tamm, 1973; Eggers, 1982). Although early experiments yielded only marginal effects of HBB on virus-infected animals, newborn mice inoculated with echovirus 9 or coxsackie virus A9 were protected by combined treatment with HBB and guanidine (Eggers, 1976). HBB alone protected coxsackie virus-infected animals, but not those infected with echovirus (Eggers, 1976).

6.4. Enviroxime (LY 122772)

Enviroxime (2-amino-1-(isopropylsulfonyl)-6-benzimidazole phenyl ketone oxime) (Fig. 25) is a benzimidazole derivative with high activity against rhinoviruses (Delong and Reed, 1980), and several human trials using enviroxime as a prophylactic agent against the common cold produced by rhinoviruses have been reported (Hayden and Gwaltney, 1982; Levandowski et al., 1982; Phillpots et al., 1983b). No clear benefit was observed (Levandowski et al., 1982), although incorporation of enviroxime into liposomes results in improved drug delivery to the lungs of mice (Wyde et al., 1988). Studies on the mode of action of this drug have been retarded because the compound is not freely available from Eli Lilly Research Laboratories for research studies.

6.5. Flavones

Flavonoids are ubiquitous compounds in the plant kingdom (Harborne, 1988). The search for antiviral agents in plant extracts led to the discovery of 3-methyl quercetin (3-MQ) from the African medicinal plant Euphorbia grantii (Van Hoof et al., 1984; Vlietinck et al., 1986) and Ro 09-0179 (Fig. 26) from the Chinese medicinal herb Agastache rugosa (Ishitsuka et al., 1982b). Analysis of the mode of action of 3-MQ indicated that it was a selective inhibitor of poliovirus RNA synthesis. At a concentration of 5 μg/mL, 3-MQ inhibited [3H]uridine incorporation in poliovirus-infected cells by 90%, whereas at 20 μg/mL, 3-MQ had no effect on cellular transcription (Castrillo et al., 1986). Analysis of the viral RNAs synthesized in the presence of this flavone showed that production of single-stranded RNA was totally blocked, whereas the synthesis of dsRNA was still detectable (Castrillo and Carrasco, 1987a). Thus, 3-MQ behaves like Ro 09-0179 in blocking the
synthesis of both plus- and minus-RNA when added from the inception of infection, because there is not enough genomic RNA to serve as a template to give rise to significant amounts of negative-stranded RNA that can be detected by hybridization (Lopez Pila et al., 1989; González et al., 1990b). Therefore, in order to test if these flavones are selective, they must be added later during infection, when sufficient amounts of plus- and minus-stranded RNA are being made. Addition of Ro 09-0179 late in infection affects viral RNA synthesis in a way that suggests a preferential inhibition of poliovirus genomic RNA (González et al., 1990b). However, a detailed understanding of the molecular mode of action of both 3-MQ and Ro 09-0179 is still lacking, because we do not know yet which viral protein is their target. It is also not known if the initiation, elongation or both these steps of viral RNA synthesis are blocked by these compounds. Moreover, we have recently found that some flavones may block early steps of poliovirus growth, suggesting that some flavones could directly interact with viral particles and block uncoating (A. Irurzun and L. Carrasco, unpublished results).

3-MQ and Ro 09-0179 inhibit poliovirus replication at concentrations 100-fold, or 1000-fold, lower than those shown for HBB or guanidine, respectively (González et al., 1990b). Ro 09-0179 selectively blocks viral RNA synthesis in poliovirus-infected HeLa cells more strongly than 3-MQ, and these findings make Ro 09-0179 a valuable compound for obtaining insight into the molecular mechanisms of poliovirus RNA replication.

Quercetin and morin are effective against mengovirus-induced encephalitis in mice when given orally (Veckenstedt et al., 1978), but these two flavonoids do not show selectivity for inhibition of poliovirus in cultured cells (Castrillo et al., 1986; González et al., 1990b). The diacyl derivative of Ro 09-0179 is absorbed better than the parent compound in the intestinal tract, making it effective against lethal coxsackie B1 infections in mice (Ishitsuka et al., 1982b).

6.6. Gliotoxin

Gliotoxin (Fig. 27) is a fungal metabolite produced by various species of Trichoderma, Aspergillus and Penicillium, and it possesses different biological activities, including antiviral effects (Suhadolnik, 1967a; Miller et al., 1968; McDougall, 1969). Gliotoxin and a number of related compounds, derived either from natural sources or by chemical synthesis, are the most potent agents that interfere with poliovirus RNA synthesis (Miller et al., 1968; Trown and Bilello, 1972). The presence of a reducing agent such as dithiothreitol prevents the inhibition of poliovirus RNA synthesis in intact cells (Trown and Bilello, 1972), suggesting that blockade by gliotoxin involves the formation of disulfide bridges between gliotoxin and essential sulfhydryl groups on the viral polymerase. The presence of dithiothreitol in cell-free systems partially lowered the gliotoxin inhibitory potential, in agreement with the idea that reducing the sulfhydryl groups abolishes the activity of gliotoxin (Trown and Bilello, 1972; Rodriguez and Carrasco, 1992).

The mode of action of gliotoxin against poliovirus has been analyzed in detail (Rodriguez and Carrasco, 1992). This fungal metabolite irreversibly inhibits the appearance of poliovirus proteins when present from the beginning of infection, but has no effect on viral translation when added later. This toxin potently inhibited the incorporation of $^3$H]uridine into poliovirus RNA soon after its addition to the culture medium (Miller et al., 1968; Rodriguez and Carrasco, 1992). Analysis of the synthesis of poliovirus plus- or minus-stranded RNA in the presence of gliotoxin indicated that this compound effectively hampered both processes (Rodriguez and Carrasco, 1992), a result which contrasts with the mode of action of other inhibitors of poliovirus RNA synthesis, such as flavones, that selectively block plus-strand RNA synthesis and suggests that the target of gliotoxin may differ from the target of flavones. Gliotoxin was a potent inhibitor of poliovirus RNA synthesis in cell-free systems using membranous crude replication complexes, a reaction that is not blocked by guanidine, HBB or Ro 09-0179. Moreover, an in vitro assay, using the purified poliovirus polymerase 3Dpol, was efficiently inhibited by gliotoxin, the first description of an inhibitor of this viral enzyme (Rodriguez and Carrasco, 1992).

Gliotoxin is rather toxic for animals, the LD$_{50}$ for mice being 7.8 mg/mL intravenously and 32 mg/mL intraperitoneally (Larin et al., 1965). Despite this toxicity, gliotoxin exerts antiviral effects in monkeys inoculated with large doses of poliovirus (Larin et al., 1965).

Antipicornavirus activities have also been described for other compounds related to gliotoxin.
These include aranotin, acetylaranotin and apoaranotin (Fig. 27), all isolated from the culture medium of the fungi *Arachniotus aureus* and *Aspergillus terreus* (Trown, 1968), and sirodesmins (Fig. 27), isolated from the fungus *Sirodesmium diversum*. Sirodesmins A, B and C differ in the number of sulfur atoms in the bridge, being two, three or four, respectively. Several hyalodendrins (Fig. 27), which also differ in the number of sulfur atoms in the bridge, were isolated from the fungi *Hyalodendrin* species and *Penicillum turbatum* (Michel et al., 1974). These compounds inhibited poliovirus, rhinovirus and coxsackie virus multiplication (Trown, 1968; Michel et al., 1974). The synthetic compound *N*,*N*'-dimethylepidithiapiperazinedione (Fig. 27) was also a very potent inhibitor of coxsackie virus RNA synthesis (Trown, 1968).

### 6.7. Aldgamycin E

A natural compound, aldgamycin E (Fig. 28), a macrolide antibiotic produced by *Streptomyces lavandulae*, inhibits the growth of poliovirus, but not of other animal viruses tested (González and Carrasco, unpublished results). A blockade of viral translation takes place when the antibiotic is present from the inception of infection, but no effect on poliovirus protein synthesis was observed when this agent was added later, suggesting that protein synthesis was not the primary target for antibiotic action. Indeed, poliovirus RNA synthesis was profoundly depressed soon after the addition of aldgamycin E. Other macrolide antibiotics, such as chalcomycin, josamycin and venturicidin, also blocked poliovirus RNA synthesis, indicating that macrolide antibiotics constitute a promising source of new antiviral agents (González and Carrasco, unpublished results).

![Chemical structures of different compounds: gliotoxin, aranotin, apoaranotin, DEPE, hyalodendrin and sirodesmin A.](image)
Fig. 28. Chemical structures of different compounds: aldgamycin E, brefeldin A, hydroxycitrate, cerulenin, oleic acid and TOFA.

6.8. Dipyridamole

Dipyridamole (Fig. 26) is a synthetic compound which is a coronary vasodilator. Dipyridamole and several of its derivatives are active against certain DNA and RNA viruses in cell culture, including mengovirus (Kuwata et al., 1977; Tonew et al., 1982). The synthesis of mengovirus RNA was completely blocked by dipyridamole under conditions where the transport of uridine was not affected, i.e. by precharging the cells with labelled uridine (Kuwata et al., 1977; Tonew et al., 1982), but more detailed analyses, using hybridization with specific probes, are necessary because dipyridamole also affects nucleoside transport.

6.9. D-Penicillamine

As indicated in Section 6.1, the action of guanidine can be antagonized by a number of amino acids, and on this basis, it was suggested that certain analogues of these amino acids might also show antipoliovirus activity (Gessa et al., 1966). D-Penicillamine (Fig. 26), a valine analogue, inhibits poliovirus growth reversibly and blocks the synthesis of poliovirus RNA at drug concentrations of 0.1–0.5 mg/mL (Gessa et al., 1966). The relative concentrations of single-stranded and double-stranded poliovirus RNA did not vary in the presence of this compound. Certainly, D-penicillamine is not a potent poliovirus inhibitor, because at a drug concentration of 1 mM, total blockade of viral DNA synthesis was not achieved (Merryman et al., 1974). The inhibitory effect of D-penicillamine is reversible and can be antagonized by several amino acids.
6.10. Coupling Membrane Synthesis with Genome Replication: Cerulenin

One of the most potent inhibitors of lipid biosynthesis in prokaryotic and eukaryotic cells is cerulenin (Fig. 28), an antibiotic produced by *Cephalosporium caerulens* (Bachrach et al., 1973). It blocks fatty-acid synthetase at the level of β-ketoacyl thioester synthetase (condensing enzyme) (Armstrong et al., 1972; Butterworth and Rueckert, 1972; Bachrach et al., 1973). If present from the initiation of poliovirus infection, there is a potent inhibition of poliovirus protein synthesis, but it has no effect on viral translation or on the proteolytic processing of the viral proteins, if added late (Guinea and Carrasco, 1990). Myristoylation of the viral protein VP4 or its precursors is not blocked by cerulenin. Cerulenin inhibits very quickly the replication of poliovirus RNA when added at any time during infection (Guinea and Carrasco, 1990), whereas it has no effect on cellular transcription. This antibiotic depresses the incorporation of labelled glycerol into phospholipids, and this inhibition parallels the blockade of viral RNA replication. The synthesis of poliovirus plus-stranded RNA synthesis is hampered (Guinea and Carrasco, 1990). Cerulenin does not have a direct inhibitory effect on poliovirus translation, indicating that the synthesis of phospholipids is continuously required for the synthesis of plus-stranded poliovirus RNA. Hence, there is some link between membrane traffic and poliovirus RNA replication.

This antibiotic is also an inhibitor of some enveloped RNA-containing viruses (Schlesinger and Malfer, 1982; Ikuta and Luftig, 1986; Pal et al., 1988). Thus, the replication of retroviruses, including HIV type 1, is blocked by cerulenin (Pal et al., 1988). It was proposed that the processing of structural proteins and the acylation of some glycoproteins could be targets for cerulenin in its inhibition of viral growth (Schlesinger and Malfer, 1982; Ikuta and Luftig, 1986; Pal et al., 1988). The inhibition of phospholipid synthesis by cerulenin also impairs the synthesis of nucleic acids in other viruses, such as EMC virus, SFV and VSV (Guinea and Carrasco, 1990; Pérez and Carrasco, 1991; Pérez et al., 1991). In fact, the synthesis of nucleic acids, not only in togaviruses, but in many other animal viruses, is closely connected to the synthesis of new membranous structures. If the synthesis of these membrane structures requires *de novo* synthesis of phospholipids, it seems plausible that inhibition of lipid synthesis would certainly lead to inhibition of viral nucleic acid synthesis (Pérez and Carrasco, 1991; Pérez et al., 1991).

6.11. TOFA and Hydroxyacetic Acid

Other compounds that block phospholipid synthesis include TOFA and hydroxyacetic acid (Fig. 28), which act in a similar manner as cerulenin (Guinea and Carrasco, unpublished results). Clofibrate, a compound that potentiates the oxidation of fatty acids, pentenoic acid, an inhibitor of fatty acid oxidation, hemicholinium, an inhibitor of choline incorporation into phosphatidylcholine, and compactine, an inhibitor of cholesterol synthesis, all had no effect on poliovirus. On the other hand, agaric acid, an inhibitor of fatty acid synthesis, sodium palmoxirate, another agent that blocks fatty acid oxidation, and 3-morpholine-1-propanol, were all too toxic in control cells to allow assay of their effects on poliovirus-infected cells (Guinea and Carrasco, unpublished results). TOFA and hydroxycitrate, two known inhibitors of lipid synthesis, blocked the appearance of poliovirus proteins when present from early stages of infection. Further analyses on the effects of these two compounds on poliovirus replication indicated that they had no action on poliovirus protein synthesis if added late during infection, whereas they potently depressed poliovirus RNA synthesis (Guinea and Carrasco, unpublished results). Furthermore, both compounds also had an inhibitory effect on phospholipid synthesis in poliovirus-infected cells. These results indicate that agents that interfere with phospholipid biosynthesis, such as cerulenin, TOFA and hydroxycitrate, hamper the replication of poliovirus RNA. Thus, the mode of action of these agents differs from that of the well-known poliovirus RNA inhibitor guanidine, because guanidine has no effect on lipid synthesis.

6.12. Membrane Traffic and Genome Replication: Brefeldin A

Membranes are continuously in motion through several compartments in eukaryotic cells (Morré and Ovtracht, 1977; Rothman and Orci, 1992; Lippincott-Schwartz, 1993). Brefeldin A (BFA)
(Fig. 28), a fungal antibiotic isolated from *Penicillium brefeldianum*, has antiviral properties (Tamura *et al.*, 1968) and blocks protein secretion and vesicular traffic (Hunziker *et al.*, 1992). It induces a rapid disaggregation of the Golgi complex, causing redistribution into the endoplasmic reticulum (Lippincott-Schwartz *et al.*, 1989, 1991; Alcalde *et al.*, 1991).

Within seconds of BFA treatment, a peripheral protein (110 kDa) of the Golgi apparatus is released in the cytoplasm and a Golgi membrane enzyme that catalyzes exchange of guanine nucleotide onto ARF is inhibited (Donaldson *et al.*, 1992; Helms and Rothman, 1992). Since transport of proteins into post-Golgi compartments in the cell is potently blocked by BFA (Klausner *et al.*, 1992), so too is the delivery of VSV G glycoprotein to the plasma membrane (Misumi *et al.*, 1986; Fujiwara *et al.*, 1988; Doms *et al.*, 1989; Oda *et al.*, 1990). In addition to VSV, an increasing number of animal viruses are reported to be affected by this inhibitor (Ulmer and Palade, 1990; Chen *et al.*, 1991; Cheung *et al.*, 1991; Whealy *et al.*, 1991). BFA inhibits the processing and transport of glycoproteins that are present in all these viruses. In this manner, enveloped viruses that mature from the plasma membrane or mature intracellularly are inhibited by this macrolide antibiotic (Ulmer and Palade, 1990; Chen *et al.*, 1991; Cheung *et al.*, 1991; Whealy *et al.*, 1991).

BFA strongly inhibits poliovirus replication (Iruzun *et al.*, 1992; Maynell *et al.*, 1992), even though this virus lacks a lipid envelope and does not encode any glycoproteins (Urzaínqui and Carrasco, 1988). Addition of BFA from the beginning of poliovirus infection blocks the synthesis of late proteins, but has no effect on p220 cleavage, indicating that the input viral RNA is translated to produce active 2A<sup>pro</sup> (Iruzun *et al.*, 1992). The presence of BFA at later times has no effect on poliovirus protein synthesis, so that this step is not a direct target for the antibiotic. Indeed, the target of BFA is viral RNA synthesis, because addition of the antibiotic at any time after poliovirus infection drastically reduces the incorporation of labelled uridine into poliovirus RNA (Iruzun *et al.*, 1992; Maynell *et al.*, 1992). Both plus- and minus-stranded RNA synthesis are diminished when BFA is present from the beginning of infection, but plus-stranded RNA synthesis is more affected when the inhibitor is added at later times (Iruzun *et al.*, 1992).

The effects of BFA on late protein synthesis by other animal viruses varies according to the virus species (Iruzun *et al.*, 1992). Among picornaviruses, rhinoviruses are sensitive to the antibiotic, whereas EMC virus is resistant. A negative-stranded RNA virus, such as vesicular stomatitis, is blocked by BFA, whereas vaccinia virus, a cytoplasmic DNA virus, is resistant (Iruzun *et al.*, 1992). It follows that even viruses that do not contain a lipid envelope and do not synthesize glycoproteins, may require newly made membranes to allow the synthesis of new genomes (Iruzun *et al.*, 1992; Maynell *et al.*, 1992). If so, the poliovirus proteins, once synthesized, will bind to membranes in the rough endoplasmic reticulum and then migrate through the Golgi complex, following the same route used by glycoproteins (Fig. 29). Once the poliovirus proteins reach the trans-Golgi network, they will be assigned to the specialized structures formed by the membranous vesicles and the RNA replication complexes (see Section 5.2). The finding that BFA immediately blocks poliovirus RNA synthesis without affecting translation suggests that RNA synthesis takes place in association with newly made vesicles and that preexisting vesicles do not support the formation of new genomes. These findings will help to clarify the action of a number of antiviral agents that interfere with the replication of genomes without affecting the viral polymerase itself. In addition, they can be exploited to devise more selective antiviral agents because, at least theoretically, we could interfere with the action of the viral proteins involved in the coupling between the membranous vesicles and the RNA replication complexes.

6.13. Oleic Acid

Addition of some fatty acids to cells in culture increases lipid synthesis (Cowen and Heydrick, 1972; Burkhardt, 1991). Conversely, unsaturated fatty acids, such as oleic acid, increase membrane fluidity and diminish the infectivity and hemolytic activity of enveloped animal virus particles. No such effects have been described for non-lipid-enveloped animal virus particles (Kohn *et al.*, 1980; Sola *et al.*, 1986a). Oleic acid (Fig. 28) blocks the synthesis of poliovirus proteins at concentrations that do not affect translation in uninfected cells (Guinea and Carrasco, 1991). This inhibition is due to a blockade of viral RNA synthesis, most probably as a consequence of modifications in the membranes connected with the RNA replication complexes. Addition of oleic acid, but not other
fatty acids, from the beginning of poliovirus infection, specifically inhibits the appearance of virus polypeptides at concentrations that do not affect translation in mock-infected HeLa cells. This inhibition is most probably due to the blockade of viral RNA synthesis, because oleic acid interferes with the synthesis of poliovirus RNA both in vivo and in cell-free systems (Guinea and Carrasco, 1991). Oleic acid increased the synthesis of phosphatidylcholine and most neutral lipids. Membranes made in poliovirus-infected cells in the presence of oleic acid differ in their buoyant density from control membranes. The incorporation of oleic acid into membranes leads to increased membrane fluidity and decreased buoyant density, making these membranes nonfunctional for RNA replication (Guinea and Carrasco, 1991). Therefore, not only is the synthesis of phospholipids required for viral RNA synthesis (Guinea and Carrasco, 1990), but the proper structure of the newly made membranes is also important to permit replication of poliovirus genomes.

7. INHIBITORS WITH UNKNOWN MODES OF ACTION

7.1. Interferons

Interferons are cellular proteins, synthesized in response to several inducers (Stringfellow, 1981; Sehgal et al., 1982), that possess antiviral activities (De Maeyer et al., 1981; Staeheli, 1990; Kerr and Stark, 1992). The interaction of the interferon molecule with the appropriate receptor on the cell surface induces the synthesis of a number of proteins, thus establishing the antiviral state (Kerr and Stark, 1992; McNair and Kerr, 1992). The precise action of these proteins against the replication of animal viruses remains largely unknown (Taylor and Grossberg, 1990).

In the case of picornaviruses, it is well established that treatment of susceptible cells with different interferon species profoundly blocks viral growth (see review by Muñoz and Carrasco, 1987). Treatment of cells with human interferon, followed by subsequent infection with poliovirus, does not prevent inhibition of host protein synthesis, indicating that translation of the input viral RNA takes place (Muñoz and Carrasco, 1983). Isolation of RNAs from poliovirus-infected cells treated with interferon indicated that the ribosomal RNAs remained intact and that the cellular mRNAs were translatable, so that degradation of cellular RNAs is not the basis of interferon action (Muñoz et al., 1983). The precise step inhibited in picornavirus growth in cells treated with interferon remains largely unexplored, despite the large number of publications investigating this effect in

Fig. 29. Diagrammatic representation of membrane flow in mammalian cells. Hypothetical sites of action for protein 2C and the inhibitors brefeldin A and flavones. E.R., endoplasmic reticulum; ERGIC, endoplasmic reticulum–Golgi intermediate compartment; TGN, trans-Golgi network.
cell-free systems and a good knowledge of the particular enzymes induced by interferon in cells (Muñoz and Carrasco, 1987). It is quite probable that the synthesis of picornaviral RNA is the step blocked in interferon-treated cells (Muñoz and Carrasco, 1983; Muñoz et al., 1983), but it is assumed by many groups that interferons primarily block viral translation with this effect being mediated by the PPP(A2'p5'A)nA (2'-5' A) synthetase and/or the P1 protein kinase upon activation by dsRNA (Taylor and Grossberg, 1990; Kerr and Stark, 1992; Zhou et al., 1993).

It is difficult to establish whether interferon primarily blocks transcription or translation, not only for picornaviruses, but also for other RNA viruses, because interference with viral RNA synthesis usually leads to a reduction in viral protein synthesis and vice versa. Nevertheless, in the case of poliovirus, translation of the input RNA is not impaired in interferon-treated cells (Muñoz and Carrasco, 1983). Moreover, human cells treated with interferon and doubly-infected with poliovirus and reovirus synthesize reovirus proteins, even though poliovirus replication is impaired (Muñoz et al., 1985a; Muñoz and Carrasco, 1987). These findings indicate that there is no indiscriminate inhibition of viral proteins in interferon-treated cells and infected with poliovirus, because these cells make reovirus proteins at control levels, even though the antiviral state against poliovirus exists in the same cells.

The mechanism underlying the blockade of picornaviruses by interferon is also unclear. If interferon blocks viral RNA synthesis, how is this accomplished? The possibility that interferon blocks translation of EMC virus or mengovirus through activation of the 2'-5' A synthetase and/or the P1 protein kinase has been investigated (Verhaegen-Lewalle et al., 1982; Rice et al., 1985; Kumar et al., 1988; Lewis, 1988). Various reports show no correlation between the activation of these enzymes and the establishment of an antiviral state, not only in picornavirus-infected cells (Silverman et al., 1982; Verhaegen-Lewalle et al., 1982; Kumar et al., 1988; Lewis, 1988), but also in other viral systems (Muñoz and Carrasco, 1987; Taylor and Grossberg, 1990). Even in the case of reoviruses that contain dsRNA as genome, there is no inhibition of viral protein synthesis in HeLa cells even though the 2'-5' A synthetase and the P1 protein kinase have been induced (Feduchi et al., 1988). Transfection of the 2'-5' A synthetase or the P1 protein kinase inhibit virus growth (Chebath et al., 1987; Coccia et al., 1990; Meurs et al., 1992; Lee and Esteban, 1993), but it remains to be determined if this inhibition is related to the mode of action of interferon, because cells transfected with the 2'-5' A synthetase gene support the growth of VSV (Chebath et al., 1987; Coccia et al., 1990). Furthermore, vaccinia virus, which is resistant to interferon blockade, is inhibited in cells transfected by the P1 protein kinase gene (Meurs et al., 1992; Lee and Esteban, 1993). The intracellular location of the 2'-5' A synthetase and the role that this enzyme plays in cellular metabolism deserve further investigation.

7.2. 2'-5' A Analogues

Cell-free systems from interferon-treated cells synthesize a series of oligo(A) compounds in the presence of ATP and dsRNA (Williams et al., 1978, 1979; Kerr et al., 1982; Kerr and Stark, 1992). These oligo(A)s characteristically have phosphodiester bonds that are 2'-5' instead of the usual 3'-5' bonds. They are collectively referred to as 2'-5' A (Fig. 30) and are synthesized in minute amounts both in normal cells and in interferon-treated cells after virus infection (Silverman et al., 1982; Laurence et al., 1984; Kerr and Stark, 1992), but they can be synthesized in higher amounts in extracts or by using the purified 2'-5' A synthetase, or even chemically (Haugh et al., 1983; Kerr and Stark, 1992). The exact role that the 2'-5' A plays in the antiviral action of interferon remains obscure, because there is no correlation between the antiviral action of interferon and the presence of 2'-5' A synthetase in cultured cells (Jacobsen et al., 1983; Lewis, 1988; Taylor and Grossberg, 1990). Apart from the role that oligo(A) could play in the action of interferon against animal viruses, direct addition of pppA2'p5'A to the cell medium prevents virus infection (Alarcón et al., 1984a). pppA2'p5'A is active against several animal viruses, including EMC virus and poliovirus, at concentrations between 0.05 and 0.5 mM. This compound does not inhibit translation; rather it blocks an early step during infection of VSV. The mode of action against picornaviruses remains undetermined (Alarcón et al., 1984a).

Since 2'-5' A is a charged molecule that does not enter easily into cells, the 2'-5' A core, or a number of derivatives, have been used to inhibit translation and to block virus infection in intact
cells (Imai et al., 1982; Eppstein et al., 1982; Haugh et al., 1983; Sawai et al., 1983; Goswami et al., 1984; Eppstein et al., 1986). The xyloadenosine analogue of 2'-5' A that is dephosphorylated at the 5'-terminus is much more stable and more active than the 2'-5' A core. Phosphorothioate analogues of 2'-5' A interfere with the activation of RNase L in HeLa cells, perhaps by competition with authentic 2'-5' A (Charachon et al., 1990). Cordycepin or 3-methyl analogues of 2'-5' A have also been synthesized. However, although these analogues had antiviral effects against several DNA-containing viruses in cultured cells, their precise mode of action against picornaviruses is not known.

7.3. Polynucleotides and Oligonucleotides

Double-stranded polynucleotide complexes mediate interferon induction, thereby exerting antiviral effects (Shugar, 1974; Sehgal et al., 1982; Shannon, 1984; Streissle et al., 1985). Apart from this action, polynucleotides themselves may have antiviral activity (Shugar, 1974; Shannon, 1984; Stebbing, 1984; Streissle et al., 1985). Thus, protection of mice against EMC virus has been observed with poly (I), tRNA or poly (C) (Stebbing et al., 1976, 1977). However, the antiviral action of these polynucleotides does not seem to be mediated by interferon induction and subsequent stimulation of the immune system, because protection was also achieved in immunosuppressed mice or athymic mice (Stebbing et al., 1976).

Other approaches used to block virus infection involve antisense RNA and ribozymes (James, 1991). Antisense oligonucleotides or oligonucleotide derivatives possess good activities against a variety of animal viruses (Stephenson and Zamecnik, 1978; Goodchild et al., 1988; Leonetti et al., 1990). Particularly effective are poly (l-lysine) conjugates (Degols et al., 1989) and phosphorothioate oligodeoxynucleotides, because they more readily enter cells and are more stable (Leiter et al., 1990; Agrawal et al., 1992). Phosphorodithioate DNA oligomers are powerful inhibitors of HIV-1 reverse transcriptase (Marshall and Caruthers, 1993). To my knowledge, this approach has not been used to block picornaviruses.

7.4. Nucleoside Analogues

Until the 1980s the synthesis of nucleoside analogues was largely directed towards inhibition of members of the Herpesviridae family. This led to the development of a most effective antiviral agent, acycloguanosine (Schaeffer et al., 1978; Elion, 1989). Research on nucleoside analogues in the last decade has concentrated on finding selective inhibitors of HIV. Some nucleotide analogues possess a broad spectrum of action and some of them also block members of the Picornaviridae family.
However, this is not a general rule because certain of these analogues, such as (S)DHPA or aristeromycin, have no effect against poliovirus (De Clercq et al., 1978, 1984). Although some nucleoside analogues may have a single viral enzyme as their site of action, some of them may affect a variety of cellular and viral enzymes (Carrasco and Vázquez, 1984). This is particularly true for adenosine (ATP) or guanosine (GTP) analogues, since these nucleosides and nucleotides participate in a variety of functions. Therefore, it can be anticipated that the action of some nucleoside analogues will be rather complex (Carrasco and Vázquez, 1984). To our knowledge, the mode of action of none of the nucleoside analogues active against picornaviruses has been analyzed in detail, although some of them, such as ribavirin (1,β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), show interesting activity.

7.4.1. Pyrazofurin, Ribavirin (Virazole), Selenazofurin and Bredinin

Pyrazofurin (3(β-D-ribofuranosyl)-4-hydroxyrazole-5-carboxamide) (Fig. 31), originally called pyrazomycin, was isolated from the fermentation broth of Streptomyces candidus. This antibiotic displayed wide-spectrum antiviral activity with a very good antiviral index. It is active against rhinovirus, and in this respect is more potent than ribavirin (De Clercq et al., 1986). An excess of uridine in the culture medium abolished the antiviral effects, suggesting that the drug blocked steps in the metabolism of nucleotides (Gerzon et al., 1971). Pyrazofurin-5’-phosphate is an antagonist of uridine metabolism and blocks orotidyl decarboxylase (Shugar, 1974; Gutowski et al., 1975), but it also inhibits 5-amino-imidazole-4-carboxamide-1-β-D-ribofuranosyl-5’-monophosphate (AICAR) formyltransferase, an enzyme involved in purine nucleotide biosynthesis. Inhibition of IMP-dehydrogenase activity by related compounds such as ribavirin has also been reported (Streeter et al., 1973; Robins et al., 1985; Yamada et al., 1988).

Ribavirin (Fig. 31) was chemically synthesized and proved to be also a wide-spectrum antiviral agent (Witkowski et al., 1972; Streeter et al., 1973; Hahn, 1979) that shows activity against HIV (Fernandez-Larsson and Patterson, 1990). This compound has good antiviral activity in several
animal models and in humans (Hahn, 1979; Hayden, 1985; Fernandez et al., 1986; Patterson and Fernandez Larsson, 1990) and has been licensed in the USA for the treatment of respiratory syncytial virus infections. Ribavirin shows antiviral activity at concentrations well below those that cause cytotoxicity (Hahn, 1979; Sidwell et al., 1979; Smith and Kirkpatrick, 1980), although the mode of action of this interesting antiviral agent remains obscure. The idea that ribavirin depletes GTP pools and/or interferes with cap mRNA formation (Goswami et al., 1979) is not supported by several lines of evidence (Smith and Kirkpatrick, 1980; Patterson and Fernandez Larsson, 1990). Picornaviruses do not possess a cap structure in their mRNA, but ribavirin potently inhibits their growth. Indeed, ribavirin is active against several members of the Picornaviridae family, including poliovirus (De Clercq, 1985; Van Aerschot et al., 1989; Andersen et al., 1992), EMC virus, FMDV (de la Torre et al., 1987), rhinoviruses and coxsackie virus (Smith and Kirkpatrick, 1980; Andersen et al., 1992). Ribavirin is the only antipicornavirus agent shown to cure cells from persistent picornavirus infections (de la Torre et al., 1987). Thus, BHK cells persistently infected with FMDV were cured after passages through medium containing low concentrations of ribavirin (de la Torre et al., 1987). Inhibition of FMDV by ribavirin in lytic infections required higher doses of the antiviral agent (de la Torre et al., 1987). Recently, the actions of ribavirin and selenazofurin against several picornaviruses have been investigated (Andersen et al., 1992). Prevention of poliovirus protein synthesis by ribavirin occurs when several rounds of virus replication take place (Andersen et al., 1992), but the drug does not inhibit poliovirus protein synthesis in a single growth cycle, even if present from the beginning of infection (Almela and Carrasco, unpublished observations).

Some compounds are structurally related to ribavirin, such as selenazofurin, that also has a broad spectrum of antiviral activity, and tiazofurin (2-fl-D-ribofuranosylthiazole-4-carboxamide) that is mainly active against some RNA viruses (Srivastava et al., 1977; Huggins et al., 1984; Kirsi et al., 1984). Bredinin, an antibiotic isolated from Eupenicillium brefeldianum, does not possess broad-spectrum antiviral activity.

7.4.2. Tubercidin, Toyocamycin and Sangivamycin

These three antibiotics (Fig. 32) are pyrrolopyrimidine nucleosides, which are adenosine analogues. They have been isolated from different Streptomyces species: Tubercidin from Streptomyces tubercidius, toyocamycin from Streptomyces toyocaensis and sangivamycin from Streptomyces rimosus (Suhadolnik, 1967b). All three were potent inhibitors of several rhinoviruses at drug concentrations below 1 mg/mL, but were toxic to cells at concentrations about 10-fold higher (Bergstrom et al., 1984; De Clercq et al., 1986). Several tubercidin analogues have been synthesized that are much more potent against coxsackievirus (Bergstrom et al., 1984; De Clercq et al., 1984). Although these compounds may have no therapeutic utility, some of them may be very useful as inhibitors of viral RNA replication, particularly for those picornaviruses, such as cardioviruses, against which no selective inhibitors of viral RNA synthesis have been described. Thus far, we do not know how these compounds affect EMC virus, nor do we know the exact step that they block in rhinovirus or coxsackie virus growth.

7.4.3. Neplanocins and Carbocyclic Adenosine Analogues

Neplanocins (Fig. 33) are carbocyclic analogues of purine nucleosides in which the ribose moiety has been replaced by a cyclopentene ring. They have been isolated from the actinomycete Ampullaria regularis. Neplanocins, as well as other carbocyclic analogues of adenosine, have been described as potent inhibitors of S-adenosyl-L-homocysteine hydrolase, resulting in the blockade of S-adenosylmethionine-dependent methylation reactions (De Clercq et al., 1984). Neplanocins A, B and C are also broad-spectrum antiviral agents that show activity mainly against DNA and (-)RNA viruses (De Clercq, 1985). These agents are good inhibitors of the coxsackie virus in Vero cells. Concentrations of 40 ng/mL of neplanocin A inhibited coxsackie B4 (De Clercq, 1985), whereas higher concentrations of these agents are required to block poliovirus or coxsackie virus in HeLa cells (De Clercq, 1985) or rhinovirus in WI-38 cells (De Clercq et al., 1991).

The carbocyclic analogues of adenosine, such as aristeromycin (C-Ado), 3-deazaadenosine (C-c3Ado) and 7-deazaadenosine (C-c7Ado) (Fig. 33), are also active against several animal viruses,
including picornaviruses. C-c³Ado fails to protect HeLa cells from poliovirus or coxsackie virus B4 infection, whereas the compound was active against coxsackie in Vero cells (poliovirus was not tested) (De Clercq and Montgomery, 1983; De Clercq, 1985). C-c⁵Ado was more active against both viruses in HeLa cells than C-c³Ado (De Clercq et al., 1984).

7.4.4. Cyclopentenylcytosine

Carbocyclic cytidine (C-Cyd) and cyclopentenylcytosine (Ce-Cyd) (Fig. 33) are broad-spectrum antiviral agents active against DNA- and RNA-containing animal viruses (De Clercq et al., 1990, 1991). Poliovirus, coxsackie virus B4 and rhinovirus 1A were not inhibited by C-Cyd, whereas they were potently blocked by Ce-Cyd (De Clercq et al., 1991).

7.4.5. 3-Deaza Guanine

3-Deaza guanine (Fig. 33), a base analogue of guanine, has good antirhinovirus activity in WI-38 cells at drug concentrations well below those that show cytotoxicity (De Clercq et al., 1986).

7.4.6. 3'-Fluoro-3'-Deoxyadenosine

Several fluorinated nucleosides have been synthesized and evaluated for their antiviral potency (Van Aerschot et al., 1989). 3'-fluoro-3'-deoxyadenosine (Fig. 32) was very active against poliovirus and coxsackie virus (Van Aerschot et al., 1989). Although the action of some of these derivatives may be related to their inhibition of S-adenosyl-L-homocysteine hydrolase, the exact mode of action of 3'-fluoro-3'-deoxyadenosine remains undetermined.
7.4.7. Cordycepin

Cordycepin (3'‐deoxyadenosine) (Fig. 32) was the first natural nucleoside antibiotic isolated. It is synthesized by Cordiceps militaris and Aspergillus nidulans and inhibits the formation of poly(A) tails in mRNAs. Cordycepin is incorporated into the 3′ terminus of several RNAs and acts as a chain terminator. The antibiotic inhibits the replication of poliovirus and rhinovirus, with inhibition of the latter being particularly sensitive (Nair and Panicali, 1976). The triphosphate inhibited incorporation of GMP by picornavirus‐specific polymerase complexes in cell‐free systems. RNA products synthesized \textit{in vitro} lacked full‐length viral RNA, indicating that RNA chain termination occurred in the presence of cordycepin (Panicali and Nair, 1978).

7.5. Other Picornavirus Inhibitors

7.5.1. Amicetin and Actinobolin

Amicetin (Fig. 34), an antibiotic produced by Streptomyces fasciculatus and Streptomyces plicatus, is an inhibitor of peptide bond formation (Vázquez, 1979). Amicetin is active in bacteria, but is less effective against mammalian cells. It inhibits both poliovirus and EMC virus at concentrations that do not affect cultured cells (Alarcón \textit{et al.}, 1984c).

Actinobolin (Fig. 34), another translation inhibitor (Vázquez, 1979), is produced by Streptomyces fasciculatus and is also an effective antiviral agent in cell culture (Alarcón \textit{et al.}, 1984c). At concentrations of 30 or 75 µg/mL, the drug protected HeLa cells from infection by either poliovirus or EMC virus (Alarcón and Carrasco, unpublished results).

Fig. 33. Chemical structures of different compounds: aristeromycin, C-c^3-Ado, C-c^7-Ado, 3-deazaguanine, neplanocin A, C-Cyd and Ce-Cyd.
7.5.2. Tetracenomycin C

Tetracenomycin C (Fig. 34) is an antibiotic produced by *Streptomyces glaucescens*. This agent blocks the growth of several animal viruses, including poliovirus and EMC virus (Alarcón et al., 1984c). Concentrations of 15 μg/mL tetracenomycin C protected HaLa cell monolayers from infection by these two viruses (Alarcón et al., 1984c).

7.5.3. Sulfated Polysaccharides

Sulfated polysaccharides and oligosaccharides inhibit viral replication at very low concentrations and show a wide spectrum of antiviral action (Alarcón et al., 1984c; González et al., 1987). These agents have no adverse effects on cultured cells even at high drug concentrations (Alarcón et al., 1984c; González et al., 1987). Some picornaviruses, such as poliovirus, are not affected by these compounds, whereas EMC virus growth is potently blocked by carrageenan or low-MW dextran sulfate (González et al., 1987). Preliminary results from our laboratory indicate that carrageenan
has no effect on protein synthesis in EMC virus-infected cells, implicating a late step of EMC virus replication as the target of these agents. This result contrasts with the action of sulfated polysaccharides on herpes virus or HIV, where there is interference with an early step of infection after interaction of the virus with the host cell receptor (González et al., 1987; Callahan et al., 1991).

7.5.4. Suramin and Trypan Blue

Suramin (Fig. 35) is a polyanionic synthetic compound that inhibits several viral and cellular polymerases (Basu and Modak, 1985), including the reverse transcriptase of RNA tumour viruses in cell-free systems (De Clercq, 1979; Jentsch et al., 1987). It shows a potent antiviral effect in cultivated cells infected with different animal viruses, including EMC virus, but not poliovirus (Alarcón et al., 1984c; Mitsuya et al., 1984; Sola et al., 1986b; Schols et al., 1990). Suramin had no effect on EMC virus protein synthesis even when present at early stages of infection, suggesting that it blocks a late step in the replication cycle (Alarcón and Carrasco, unpublished results). Trypan blue, a very nonpermeant dye, also protected cell monolayers from viral infection (Alarcón et al., 1984c), as occurs with other charged compounds (Balzarini et al., 1986). In contrast with trypan blue, high concentrations of suramin had no adverse effects on either cell morphology or cell growth, even after prolonged incubation. Mice infected intravaginally with HSV-2 survived when treated with suramin (Alarcón and Carrasco, unpublished results).

7.5.5. HPA 23

The mineral condensed ion ammonium 5-tungsto-2-antimonate (HPA 23) is a heteropolyanion that has a broad spectrum of antiviral activity and blocks EMC virus (Werner et al., 1976; Machamer et al., 1992). A single intraperitoneal injection of HPA 23 protected mice from EMC virus (Werner et al., 1976). Since this compound is a polyanion that also blocks reverse transcriptase (Chermann et al., 1975; Ablashi et al., 1977), it is possible that its mode of action may be similar to that of sulfated polysaccharides.

![Chemical structures of different compounds: suramin and trypan blue.](image)
7.5.6. Glycyrrhizinic Acid

Glycyrrhizinic acid (Fig. 34) is a natural compound extracted from the roots of Glycyrrhiza glabra. This compound has a broad spectrum of antiviral action (Pompei et al., 1979; Alarcón et al., 1984c) and blocks EMC virus, but not poliovirus, growth (Pompei et al., 1979). Addition of this agent from the beginning of EMC virus infection allows the synthesis of viral proteins at control levels, whereas the formation of infectious and physical particles is severely diminished (Lacal and Carrasco, unpublished observations). It is possible that this agent blocks the assembly of new EMC virions, but such an action has not yet been demonstrated.

7.5.7. Tenuazonic Acid

The antibiotic tenuazonic acid (Fig. 36) was reported as an inhibitor of poliovirus growth (Miller et al., 1963). It was later found that this compound blocks protein synthesis in mammalian cells, and it was, in fact, the first inhibitor of eukaryotic ribosomes to show selectivity in the blockade of human, as opposed to yeast ribosomes (Carrasco and Vázquez, 1973).

7.5.8. N-Phenyl-N'-Arylthiourea

Derivatives of N-phenyl-N'-arylthiourea (Fig. 36) are inhibitors of coxsackie virus, poliovirus, FMDV and rhinoviruses. Drug concentrations of approximately 2 μg/mL of N-phenyl-N'-3-hydroxyphenyl-thiourea (PTU-23) reduced rhinovirus growth by one-log (Galabov et al., 1977). The potency and antiviral index of N-phenyl-N'-arylthiourea are rather low and probably these compounds block an early step of rhinovirus replication after adsorption (Galabov et al., 1977; Galabov, 1979). PTU-23 had activity against coxsackie infection in mice, whereas another derivative (PTU-24) was active against FMDV in mice and guinea pigs (Galabov, 1979).

7.5.9. Dichlorobenzoate (M12325)

5-Aminosulfonyl-2,4-dichlorobenzoate (M 12325) (Fig. 36) inhibits several species of RNA-containing viruses, including echovirus and rhinovirus, but has no effect against DNA viruses (Ohnishi et al., 1982). It has a good antiviral index in cultured cells and is effective in mice against influenza virus (Ohnishi et al., 1982).

7.5.10. G413

2-Amino-5-(2-sulfamoylphenyl)-1,3,4-thiadiazole (G413) (Fig. 36) belongs to a series of derivatives that block DNA and RNA viruses (Bonina et al., 1982). Drug concentrations above 10 μg/mL inhibited poliovirus, echovirus and coxsackie virus. It has been suggested that G413 may bind to structural proteins and prevent virion assembly (Bonina et al., 1982), although more profound studies on this agent are required to obtain a firm conclusion of its mode of action.

7.5.11. Thiazolinone Derivatives

A series of water-soluble thiazolinone derivatives (IMTO) (Fig. 36) exhibit antiviral properties. The N-(2-dimethylamino-2-methylpropoxycarbonyl) derivative was effective against influenza and coxsackie viruses in cultured cells (Harnden et al., 1979).

7.5.12. Mycophenolic Acid

Various species of the genus Penicillium produce mycophenolic acid (Fig. 37). This antibiotic blocks the replication of several animal viruses, including some retroviruses (Williams et al., 1968), EMC virus and coxsackie virus (Ando et al., 1968; Cline et al., 1969; Planterose, 1969). It is still uncertain if this blockade is due simply to cellular toxicity.
7.5.13. Didemnins

A new class of depsipeptides, didemnins (Fig. 37), were isolated from a Caribbean tunicate (sea squirt, family *Didemnidae*) that had effects against coxsackie virus (Rinehart and Gloer, 1981). Although some of these compounds, particularly didemnin B, had a potent antiviral effect in cell culture, their cytotoxicity was very high and, hence, the antiviral index was poor (Rinehart and Gloer, 1981).

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**Fig. 36.** Chemical structures of different compounds: tenuazonic acid, N-phenyl-N’-4-hydroxyphenyl thiourea, mycophenolic acid, G 413, IMTO and M 12325.

**Fig. 37.** Chemical structures of different compounds: didemnin A, atropine, rifampicin, DIQA, papaverine and dehydroemetine.
7.5.14. Atropine

Atropine (Fig. 37) is an antiviral substance extracted from *Atropa belladonna*. This agent inhibits the growth of several animal viruses (Alarcón et al., 1984b), including poliovirus, but not EMC virus (Alarcón and Carrasco, unpublished results).

7.5.15. DIQA

3,4-Dihydro-1-isouquinolineacetamide hydrochloride (DIQA) (Fig. 37) and some related compounds, dehydroemetine and papaverine, show some antiviral effects against picornaviruses (Grunberg and Prince, 1968, 1970). Only DIQA (0.1 mg/mL) was active against coxsackie virus in tissue culture (Grunberg and Prince, 1970). Studies on the mode of action of this agent indicated that it blocked an early step of Columbia SK virus growth, different from adsorption of the virus to L cells (Murphy and Glasgow, 1970). Probably, this early step is the entry of virions into cells (Murphy and Glasgow, 1970). Clinical trials with DIQA have been conducted to prevent rhinovirus infection in volunteers (Togo et al., 1973a). Oral administration did not prevent the development of colds, but the symptoms in the treated group were milder than in the placebo-treated group (Togo et al., 1973a).

7.5.16. Rifamycins

Rifamycins A–E have been isolated from the broth of *Nocardia mediterranei*. Rifampicin is a chemical derivative, 3-(4-methylpiperazinoiminomethyl) rifamycin B (Fig. 37). These antibiotics and several derivatives have activity against several animal viruses (Gurgo et al., 1982). FMDV and poliovirus growth is inhibited by rifampicin in suspension culture, but not in cell monolayers (Grado and Ohlbaum, 1973). Rifampicin was equally effective when added before or after virus entry. The specificity and reproducibility of this inhibition is obscure, because the same authors also found inhibition of these viruses by actinomycin D (Grado and Ohlbaum, 1973).

7.5.17. Antibiotic K-582

K-582 is a peptide antibiotic isolated from the culture filtrate of *Metarhizium anisopliae*, which exhibits antiviral activity against polio, influenza and Newcastle disease virus, perhaps by inducing an antiviral substance related to interferon (Sakai et al., 1983).

7.6. Undefined Agents

Several undefined agents have been reported to block picornaviruses. Indeed, antiviral researchers are well acquainted with a numerous list of publications claiming antiviral effects with extracts, exudates, secretions, etc., from the most diverse organisms. In many instances, these crude preparations (or even purified components) owe their “antiviral activity” simply to toxicity. In some cases, however, some specific antiviral effects have been documented. Thus, helenine, perhaps a ribonucleoprotein substance, produced by *Penicillium funiculosum*, has effects against Columbia SK virus and SFV (Shope, 1953a, b, c).

Extracts from different plants and fungi can interfere with the growth of poliovirus, coxsackie virus and echoviruses in cultured cells (Goulet et al., 1960). Treatment with neurotoxoids from cobra venom interferes with poliovirus infection in monkeys and cultured cells (Sanders et al., 1953, 1958; Miller et al., 1977). Abalone (*Haliotis refescens*) is a marine animal commonly used as food in China, and its juice has some inhibitory effects on mice infected with poliovirus (Li, 1960). Apple juice, grape juice and wines are virucidal for some viruses, including poliovirus (Konowalchuk and Speirs, 1953, 1958; Miller et al., 1977). A lipid component of milk inhibits viral growth (Sabin and Fieldsteel, 1962; Matthews et al., 1976). The same occurs with acidic substances from pine cones and pine seed shell extracts (Fukuchi et al., 1989a; Mukoyama et al., 1991b) and with tea extracts (Green, 1949; Mukoyama et al., 1991a). These effects perhaps relate to tannin and
polyphenol content, substances that are known to be antiviral agents (Green, 1948; Kucera and Herrmann, 1967; Fukuchi et al., 1989b).

Finally, a number of antiviral compounds have been reported to have a negligible or no effect against several picornaviruses. Such compounds include camptothecin (Horwitz et al., 1972), gossypol (Dorsett et al., 1975) and hypericin (Tang et al., 1990).

8. CONCLUSIONS AND FUTURE PROSPECTS

Picornaviruses represent one of the best understood animal virus groups in molecular terms. They were the first animal viruses containing an RNA genome that was sequenced, the first animal virus particles to be solved at atomic resolution and the first animal viruses to be totally synthesized in the test tube (Harber and Wimmer, 1993). The analysis of the picornavirus replication cycle, in many instances, guides work with other virus groups, although picornavirus biology also shows peculiarities typical of this virus group, e.g. the internal initiation of translation on picornavirus mRNA (Meerovitch and Sonenberg, 1993).

Picornavirus inhibitors have very much aided in the elucidation of the different steps of the picornavirus replication cycle at the molecular level. The growing armamentum to block given steps of picornavirus growth provides useful tools to investigate details of the molecular biology of picornaviruses. In addition, these studies provide, in some instances, new approaches to develop antiviral agents. This is the case of the recently found connection between picornavirus genome replication and membrane traffic in mammalian cells (Guinea and Carrasco, 1990; Irurzun et al., 1993), or the finding that picornaviruses encode for viroporins (Lama and Carrasco, 1992b), viral proteins that make pores in the membrane and modify membrane permeability (Carrasco et al., 1993), allowing the passage of nonpermeant inhibitors into virus-infected cells (Carrasco, 1978).

The fact that some picornaviruses cause disease in humans and in animals prompted the investigation of more effective and nontoxic compounds to inhibit their replication. The elegant studies on agents that bind to virion particles and block their uncoating exemplifies not only one of the most refined analyses on the mode of action of antiviral agents, but also provides promising compounds to effectively attack the common cold in humans (Rossmann and Johnson, 1989; McKinlay et al., 1992).

Finally, the growing interest in picornavirus inhibitors will expand the number of new antipicornavirus agents and will help to unravel the modes of action of many of them. I predict that future studies to broaden our knowledge of the exact mechanism of action of these inhibitors will provide more surprises to the molecular biology of this ever-interesting group of viruses.

Acknowledgements—I wish to thank those who sent me their reprints and preprints, and I look forward to receiving reprints from those whose work has not been adequately covered in this review. I should like to thank Ms Carmen Hermoso for her help with typing and Mr José María Galán for his good artwork with the figures. During the writing of this review, our laboratory was financed by grants from Plan Nacional project number (BIO 92-0715), DGICYT project number PB90-0177 and an institutional grant from Fundación Ramón Areces.

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