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Review

Antivirals against animal viruses

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ABSTRACT

Antivirals are compounds used since the 1960s that can interfere with viral development. Some of these antivirals can be isolated from a variety of sources, such as animals, plants, bacteria or fungi, while others must be obtained by chemical synthesis, either designed or random. Antivirals display a variety of mechanisms of action, and while some of them enhance the animal immune system, others block a specific enzyme or a particular step in the viral replication cycle. As viruses are mandatory intracellular parasites that use the host's cellular machinery to survive and multiply, it is essential that antivirals do not harm the host. In addition, viruses are continually developing new antiviral resistant strains, due to their high mutation rate, which makes it mandatory to continually search for, or develop, new antiviral compounds. This review describes natural and synthetic antivirals in chronological order, with an emphasis on natural compounds, even when their mechanisms of action are not completely understood, that could serve as the basis for future development of novel and/or complementary antiviral treatments.

Contents

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1. Introduction

Antivirals are antimicrobial compounds, either produced by living organisms or obtained by chemical synthesis, that inhibit viral replication. Antivirals interfere with one or more of the viral life cycle stages (Fig. 1), this includes: cell attachment (Fig. 1.1), cell penetration (Fig. 1.2), viral uncoating (Fig. 1.3), viral genome (DNA/RNA) replication (Fig. 1.4), maturation (Fig. 1.5) and viral progeny release (Fig. 1.6). Treatment with antivirals can stop viral infections and, thus, these compounds represent an important tool to complement the action of vaccines [1]. Unfortunately, antiviral therapies do not represent what Ehrlich referred to as the "magic bullet", as they can cause serious side effects to the host. Viruses use the host machinery for their survival and replication; hence

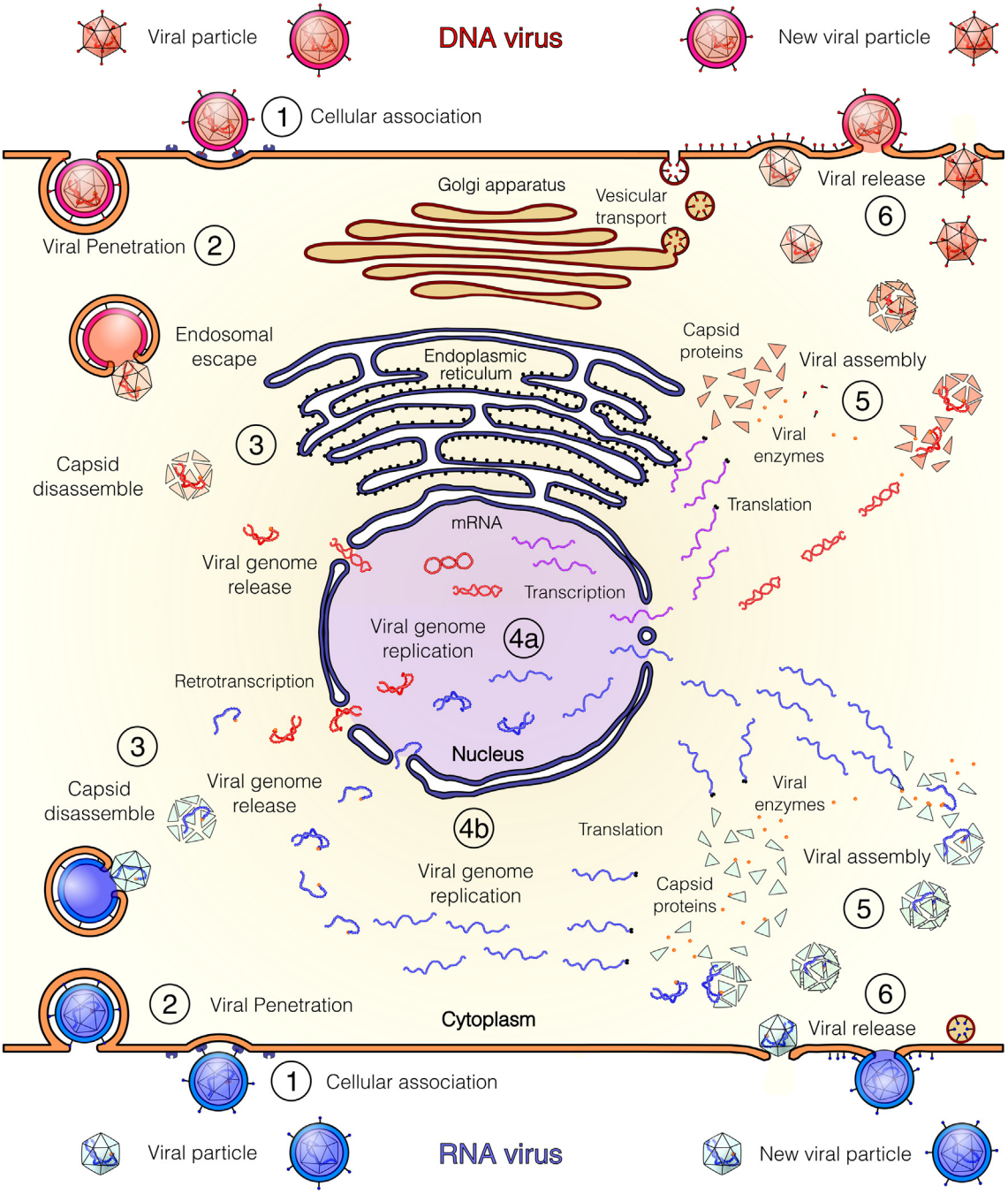

Fig. 1. Schematic representation of viral life cycle. Top: DNA virus cycle (red), bottom: RNA virus cycle (blue). The numbers indicate the steps usually targeted by antiviral compounds: 1. Adsorption, 2. Internalization, 3. Viral capsid release and disaggregation, 4. DNA/RNA replication (a: nucleus, b: cytoplasm), 5. Viral assembly and maturation, and 6. Release of mature virions.

antivirals can have deleterious effects on the host's cells. We currently envisage antivirals as compounds capable of stopping viral replication, giving the patient's immune system more time to neutralize and eradicate the viral pathogen. Antiviral compounds were initially used only in palliative care, while the illness followed its natural course; but true antivirals started to be used in the 1960s. The plant flavones and flavonoids were some of the first compounds with antiviral activity described [2], as well as several quinone and isoquinoline derivatives [3]. Other early antivirals include phenanthridine compounds, twenty-three of which are active on influenza A virus, while seven display suppressive action on Rous sarcoma virus infecting chicks [4]. But, after those early years, antiviral research concentrated on three classes of compounds, substituted benzimidazole ribosides, dicarbonyls and thiosemicarbazones. These antivirals have since remained of medical importance, and are regularly the subject of reviews. One such review was published by Hurst and Hull in 1956 [5], and included 478 references, but unfortunately, these early antimicrobials do not have clinical interest. It soon became apparent that these compounds, although efficient against viruses, were not the panacea to control viral infections, since they could be effective against a particular virus, but not against closely related organisms. In the early years of antiviral research a number of compounds were used in an attempt to control viral diseases, these include the urazoles and related compounds [6], and the 3-heterocyclic azo-4-amino-(or-hydroxy)-naphthalenesulfonic acids and their derivatives (acidic polymers that inhibit cellular ribonucleases [7]). Plant extracts were also used as antivirals, partly due to the influence of Chinese medicine. One such example is aristolochic acid, produced by the Aristolochiaceae, aka birthwort, family of plants that was already used by the Greeks and Romans to treat urinary problems and snake bites. The protective action of aristolochic acid was experimentally demonstrated in 1980 in rabbit eyes infected with herpes simplex [8], but this compound was classified by the Food and Drug Administration (FDA) as a potential toxic substance, due to its carcinogenic and mutagenic activities, and denied approval as a food supplement. The most common strategy in the search for antiviral drugs in nature is to make extracts from plants, animals, fungi, and bacteria and assay them on a variety of viruses. In this way, Woo and colleagues [9] tested more than 300 plant extracts against Herpex simplex virus types I (HSV-1) and II (HSV-2).

Unfortunately, the antiviral drugs thus obtained are usually toxic to mammals and, as reported by Lampis and coworkers in 2001 [10], "*their use is still limited to cases where benefits exceed the damage, or when drug toxicity is too low to cause harmful effects*". The use of synthetic antiviral drugs in combination with natural antibacterial and antiviral compounds, such as lysozyme, could result in unexpected benefits, such as increasing the viral range of action and decreasing the therapeutic dose, as well as reducing the occurrence of antiviral-resistant strains [11,12]. Antivirals are designed to block a particular step in the viral life cycle, with the end result that no viral progeny is generated. Viruses however, exhibit a high mutational rate, which favors the appearance of resistant viral strains, thus limiting the usefulness of the antiviral compound. For example, the seasonal influenza virus strains incorporate mutations in the neuraminidase protein (H274T), thus making it resistant to antivirals such as Tamiflu (Oseltamivir, CID: 65028) [13].

We also describe here the different ways of obtaining antiviral compounds, including a variety of natural sources. These natural compounds must be envisioned as complementary to chemically synthesized antivirals with two main purposes: i) lower the required therapeutic dose, and ii) decrease the frequency of emerging antiviral-resistant viral strains.

2. Antivirals from animal cells

Although the peptides and proteins reviewed here are structurally very heterogeneous, all of them contain a positively charged domain that allows the compound to adopt an amphipathic conformation that locates them at hydrophilic/hydrophobic interfaces. This amphipathic conformation is believed to be a prerequisite for the antimicrobial activity, and it ultimately results in cell membrane disruption [14]. In addition, many of these proteins can be degraded into shorter peptides (perhaps the best example of this is lactoferrin; see Section 2.2.2.2) with enhanced antiviral activity [15,16].

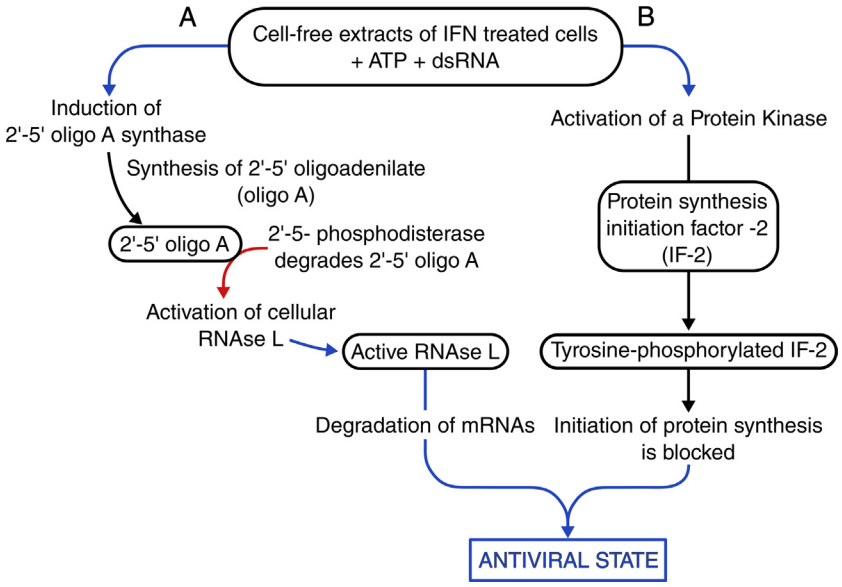

Fig. 2. Summary of how cells attain an antiviral state, which requires two signaling pathways: A) Results in the activation of RNase L, which degrades viral and cellular mRNAs. The oligoadenylate synthase (OAS) can use up to 97% of the available ATP to synthesize 2′-5′ oligoadenylate. The enzyme 2′-5′ phosphodiesterase activates RNase L, but can also cleave the 3′end of tRNAs. The resulting activated RNase L can then hydrolyze single-stranded RNAs, including some cellular mRNAs, although it is unable to hydrolyze double-stranded RNA. B) Protein Kinase phosphorylates major proteins, such as the α-subunit of initiation factor 2 (IF-2), and phosphorylated IF-2 blocks eukaryotic protein synthesis.

2.1. The interferons

Interferons are proteins produced by animal cells that inhibit virus replication. Isaacs and Lindenmann [17] reported, in 1957, that embryonic hen eggs challenged with orthomyxoviruses produced a substance that they called "interferon". This novel discovery opened up a new line of research in virology, and it is currently accepted that the ability to produce viral interfering proteins was acquired late in vertebrate evolution. This also created a dichotomy in viral interference: i) viral interference mediated by interferon, and ii) viral interference not mediated by interferon. The years 1958 and 1959 saw the publication of classic papers studying the mode of action and distribution of these proteins, that were found to be produced by many animals [18–21].

Today, interferons (IFNs, with more than 20 genes so far described) are considered signaling proteins (cytokines) that can activate the immune system through specific pathways (activation of natural killer cells and macrophages, increasing expression of the major histocompatibility complex antigens) in order to eliminate infecting viruses (or even tumors). Interferons are synthesized by higher eukaryotes, and highly coiled RNA is mainly responsible for inducing the synthesis of these proteins (Fig. 2). The IFNs currently known can be grouped into three categories: Type I IFN, Type II IFN, and Type III IFN. Type I IFNs (formerly known as acid-resistant) are glycosylated or phosphorylated proteins, produced by induced leukocytes, lymphoblastic cells and fibroblasts [22]. This group includes subtypes IFN-α, IFN-β, IFN-δ, IFN-ε, IFN-ζ, IFN-κ, IFN-ν, IFN-τ, and IFN-ω [23,24].

Type II IFN (also known as immune interferon or γ interferon) is produced by induced lymphoid cells, lymphokine-activated macrophages [25] and T-lymphocytes activated by interleukin-12 [26,27]. In humans, this is an acid-labile lipoprotein spanning 166 amino acids, encoded by a single gene [28], whereas the mouse counterpart only has 155 amino acids. This type of interferon, however, can be induced by interleukin-unrelated compounds, such as phytohemagglutinin, concanavalin A and tuberculin; these compounds have been shown to trigger interferon synthesis in cultured mouse spleen cells [29–31].

Type III IFN was originally described, in 1965 [32], as an antiviral protein and later re-discovered in the early 21st century [33,34]. Although the activity of this IFN group is less characterized than that of the type I and II molecules, it is known Type III IFNs are encoded by four genes [23,28]. Both type I-and type III-IFNs are activated following the detection of microbe-associated molecular patterns (MAMPs [35,36]), molecules that are recognized by cells of the innate immune system through Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs), as reported by Stetson and Medzhitov in 2006 [37] and Monroe and colleagues in 2010 [38]. This recognition triggers phosphorylation of several transcription factors, then translocated into the cell nucleus; these include the interferon regulatory factors (IRFs). In fact, IRF1, IRF3, IRF4, IRF5, IRF7, and IRF8 play an important role in the transcription of IFN-α genes. These IFRs contain a conserved N-terminal region, spanning ca. 120 amino acids, that specifically binds to the interferon consensus sequence, located upstream of the interferon genes [39].

2.2. Antiviral proteins other than interferons

2.2.1. Lysozyme

Lysozyme (EC. 3.2.1.17; CAS: 9001-63-2) is a ubiquitous enzyme originated some 400–600 million years ago. It constitutes an integral part of the innate immune system in multicellular organisms, and was first reported by Nicolle in 1907 [40] and Laschtschenko in 1909 [41], although it was named lysozyme by Alexander Fleming in 1922 [42]. This is an intriguing small enzyme, human lysozyme has a molecular weight of ca. 15 kDa, although milk lysozyme can reach 18 kDa, and the paradigmatic hen egg white lysozyme (HEWL) is only 14.3 kDa. Lysozyme is one of the most intriguing molecules and carries out a variety of functions in the metabolism of living creatures, ranging from prokaryotic organisms to higher eukaryotes. It was the first protein to be fully sequenced [43], and the human gene was found to be located in chromosome 12 and contain four exons [44], as it is the case for HEWL (Fig. 3) [45]. Lysozyme can be present in nasal mucus, tears, saliva, and in gastric, duodenal, ileal, and colon secretions, and even in the cerebrospinal fluid of human infants [46,47]. Although this enzyme is defined as a 1,4-β-D-N-acetyl muramidase that can hydrolyze the glycosidic linkages present in bacterial peptidoglycan, lysozyme can also activate bacterial autolysins [48], act as a chitinase [49], and exhibit antiviral activity [50–52]. In addition, the amount of this protein in the human gut can drastically

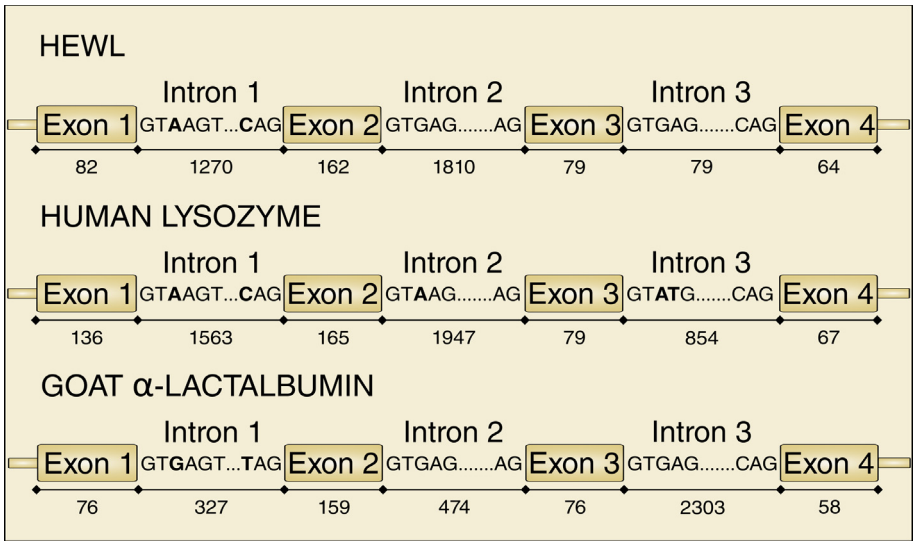

Fig. 3. Structural comparison of the genes encoding hen egg white lysozyme (HEWL), human lysozyme (GenBank: X14008.1) and goat α-lactalbumin genes, modified from Kumagai et al. [45]. The boxes represent the protein-coding exons and the numbers indicate the nucleotide base pairs in both exons and introns; only the areas coding for the mature protein are depicted in the figure. The mutational transversions (A ↔ G and C ↔ T) and transitions (T ↔ A) are found in introns on genes.

vary, depending on the emotional estate of the person [46]. Apart from exhibiting antibacterial activity, lysozyme can, under certain circumstances, control the development of animal viruses, such as orthomyxoviruses [53].

In summary, lysozymes are grouped into types according to their sequence similarities. These groups include: "chicken-type or HEWL" (c-lysozyme and stomach lysozymes), "goose-type lysozyme or goose egg-white lysozyme (GEWL)" (g-lysozyme), "T4 phage-type lysozyme" (or phage type), "bacterial lysozyme", "insect-type lysozyme", and "plant-type lysozyme". Weaver and co-workers stated in 1984 [54]: *"GEWL and HEWL have in common parts that have no counterparts in T4L. Conversely, GEWL and T4L have common structural elements that do not occur in HEWL. This pattern of structural similarity could easily have arisen through divergent evolution from a common precursor, but would not be expected to have resulted from independent events during evolution*". Although all lysozymes display antiviral activity, this action appears to be independent from their bacteriolytic activity, and is related to their positive charge [55] that induces instability in cellular membranes, as demonstrated by Cisani and colleagues in 1989 [56]. These authors found that, while HEWL stops the typical cell fusion induced by herpes simplex, addition of negative charges to this molecule drastically reduced its antiviral activity [57]. Lysozyme is active not only against HSV1 (including anti human herpes simplex-8 virus), but also against human immunodeficiency virus (HIV)-1 [51,58]. The use of lysozyme as an antiviral is potentiated by the fact that there is a very low risk of the protein being toxic, even in an overdose. Cerven and coworkers [59] studied the effect of a lysozyme overdose in rats and were unable to identify any evidence of toxicity caused by the enzyme. Indeed, they found no signs of differences in the biological parameters of animals fed up to 360 mg/kg/day of recombinant human lysozyme, as compared to untreated counterparts.

2.2.2. Antiviral proteins of milk

Undoubtedly, milk is a unique resource for translational medicine and, as recently described [60], it contains a rich pool of biologically active molecules with demonstrated clinical benefits, which will have medical applications in the future. Many of such peptides and proteins have antiviral activity and are included in the sections below.

2.2.2.1. α-lactalbumin. α-Lactalbumin (LALBA, a non-enzymatic Ca^{+2} binding protein, Fig. 4A) is another milk protein of importance, and, in addition to c-lysozyme and calcium-binding lysozyme [61], belongs to the lysozyme gene family. Regarding the antimicrobial role of lysozyme in exocrine secretions in mammals, we would like to refer the reader to the essential review by Mckenzie and White in 1991 [62]. C-lysozyme and LALBA share a similar primary sequence and structure (Fig. 4), indicating that they probably evolved from a common ancestral protein [63]. Approximately 40% of the amino acids are conserved in both proteins, although their functions are different; as indicated above, LALBA has the rare ability among lysozymes to bind calcium [64]. After binding calcium, LALBA is involved in the synthesis of lactose in mammals, while the enzyme present in the gut plays a role in tumor apoptosis [65]. LALBA not only has antiviral, antitumor and anti-stress properties, but has also been described as an anxiolytic, and even as a compound that lowers blood pressure and can prevent diarrhea (for a review see Zimecki and Kruzel [66]). This milk protein (or its fragments), displays an enhanced antiviral activity when modified by simple radicals, such as 3-hydroxyphthalic anhydride. Berkhout and colleagues [67] showed that 3-hydroxyphthalic anhydride-modified LALBA potently inhibits HIV-1 replication (Fig. 1.3), and concluded that HIV inhibition is a general property of negatively charged polypeptides. This was further confirmed by Oevermann and coworkers [68], who reported in 2003 that, of the different types of viruses tested, herpes simplex virus type 1 (HSV-1) was by far the most sensitive to these modified compounds. In addition, recent investigations indicate that both methylated LALBA and its peptic hydrolysates have anticytomegaloviral activity [69].

LALBA, as is the case for lysozyme, can efficiently inhibit HSV viruses, either as a full-length protein or when digested into tryptic peptides. HSV viruses cause a variety of clinical syndromes, such as herpetic keratitis (an important cause of infective blindness), encephalitis, and even miscarriage (through transplacental viral transmission to the fetus). HSV are large, enveloped, nuclei-replicating viruses that can be chemically treated using nucleoside analogs such as acyclovir [9-(2-hydrorulteoxymethyl) guanine] (see Section 7.4) [70,71]. HSV strains do, however, often develop antiviral resistance, through mutations in genes coding for thymidine kinase or DNA polymerase [72]. Sitohy and colleagues [73] demonstrated that either methyl-esterified α-lactoalbumin, or the tryptic peptides derived from it, are stronger antivirals against HSV viruses than their unmodified counterparts. The authors proposed that this antiviral activity could involve one, or several, of the following mechanisms: (i) disruption of the viral envelope, or capsid protein constituents, compromising capsid integrity (Fig. 1.3); (ii) restraining the interactions between viral and cellular proteins essential for viral infectivity (Fig. 1.1); (iii) the compound binding to the negatively charged viral phosphoprotein VP16 (Fig. 1.1), inhibiting the transcriptional activities of the immediate early genes (Fig. 1.4); (iv) the antiviral attaching to viral DNA, preventing it from transcription and replication (Fig. 1.4); and (v) inhibiting the hydrolytic activity of the herpes virus protease, essential for capsid formation (Fig. 1.5) [73]. Human LALBA was recently successfully cloned and expressed in transgenic pigs [74], and this recombinant protein efficiently inhibited dipeptidyl peptidase-IV activity, suggesting that α-lactoalbumin could serve as a natural precursor for a dipeptidyl peptidase-IV inhibitor, and could therefore be used in the control of unwanted viral populations in the intestine of suckling piglets.

2.2.2.2. Lactoferrin. Lactoferrin (Lactotransferrin, P02788) is a multifunctional 80 kDa glycoprotein, secreted as an innate immunity factor, often found alongside lysozyme in human secretions (saliva, milk, colostrum, etc.). This protein is also synthesized in specific granules inside neutrophils [75,76]; thus it is part of the inflammatory response, generated by mucosal epithelium and polymorphonuclear leukocytes, released from the granules of neutrophils in response to microbial infections (including viruses such as HIV-1 or Hepatitis C virus (HCV)) [77–79]. Superti and coworkers demonstrated in 1997 [80] that Fe^{+3}-lactoferrin can inhibit rotavirus replication, with apo-lactoferrin as the most active derivative, by preventing primary virus attachment to mammalian cells (Fig. 1.1). Lactoferrin is a potent inhibitor of HIV-1 reverse transcriptase, with an IC_{50} near 6 μM [81]; it also efficiently inhibits hepatitis C infection, by preventing the binding of viral ligands to their cellular receptors (Fig. 1.1) [82]. In addition, lactoferrin-derived peptides can have enhanced antiviral properties [83]. Lactoferrin was first isolated by Sorensen and Sorensen [84] from bovine milk, and it is a non-haem iron-binding peptide that belongs to the transferrin family of proteins [85]. Lactoferrin has been reported to inhibit respiratory syncytial virus, hepatitis B virus (HBV), adenovirus, poliovirus, hantavirus, Sindbis virus, Semliki forest virus, echovirus, enterovirus, and rotavirus [86]. Ng et al. also studied the antiviral activities of whey proteins [87] and found that some of them could inhibit the HIV reverse transcriptase (Fig. 1.3). The HIV-1 protease and integrase are also inhibited by casein, bovine LALBA and β-lactoglobulin, while both methylated-LALBA and methylated-β-lactoglobulin can prevent

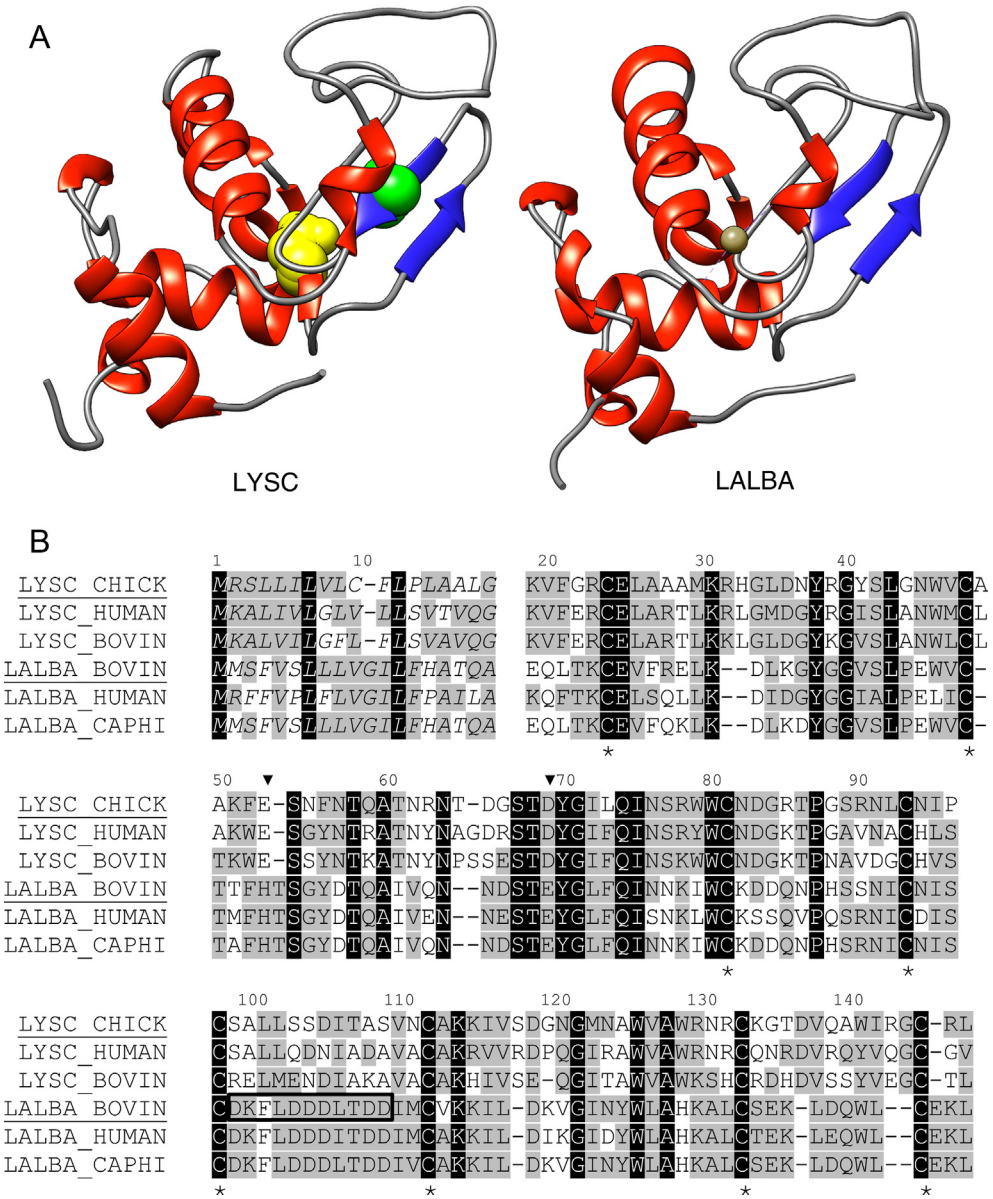

Fig. 4. A. Three-dimensional structures of the hen egg-lysozyme (LYSC) and the bovine α-lactalbumin (LALBA). The molecular models were obtained from the Protein Data Bank (PDB; http://www.rcsb.org/pdb; 1JPO and 1HFZ respectively) and the molecular graphics were performed using the UCSF Chimera package (http://www.cgl.ucsf.edu/chimera). The amino acids constituting the active site of LYSC are depicted as spheres and represent residues Glu_{35} (yellow) and Asp_{52} (green) of the mature protein. The calcium atom in the LALBA molecule is shown as a sphere. B. Amino acid sequence alignment (using Vector NTI AlignX) for hen egg-lysozyme (LYSC_CHICK; P00698), human lysozyme (LYSC_HUMAN; P61626), bovine lysozyme (LYSC_BOVIN; P04421), bovine α-lactalbumin (LALBA_BOVIN; P00711), human α-lactalbumin (LALBA_HUMAN; P00709), and goat α-lactalbumin (LALBA_CAPHI; P00712). The residues are numbered according to the LYSC_CHICK sequence. Signal peptides (indicated in UniProt; http://www.uniprot.org/) are shown in italics. Identical residues are marked in black, while similar and conserved residues are in grey. The asterisks below the sequences identify cysteine residues involved in disulfide bonds. The black triangles above the sequences indicate the LYSC_CHICK active-site residues. Calcium binding residues in bovine α-lactalbumin are indicated with a rectangle.

the development of cytomegalovirus diseases. In addition, either methylated-LALBA or methylated and ethylated β-lactoglobulins can prevent HSV. Finally, esterified beta-lactoglobulin and esterified lactoferrin can control infections caused by certain orthomyxovirus strains (Influenza A, H5N1). Swart and colleagues [88] reported that both native lactoferrin and its acylated form can bind to the V3 loop of the gp120 envelope protein of HIV-1 and HIV-2 (Fig. 1.1), and this represents the most likely mechanism of action of these compounds against HIV viruses. Other viruses, such as the lethal avian influenza A (H5N1) virus, are also susceptible to these proteins [89].

2.2.2.3. Bovine lactoferricin and lactoferrampin. Bovine lactoferricin was the first antimicrobial peptide isolated from lactoferrin (Lactotransferrin, P24627) by pepsin digestion [90,91]; this generated a 25-residues long cationic disulfide cross-linked peptide. The human lactoferricin counterpart is much larger (49 amino acids) and displays lower biological activity than its bovine counterpart [92]. Bovine lactoferrampin is a peptide originally suspected to have antimicrobial properties by Hoek and colleagues, in 1997 [93], and finally described as an antimicrobial compound by van der Kraan and co-workers in 2004 [16]. This 17-residue peptide spans amino acids Trp_{268}-Arg_{284} of bovine lactoferrin and, although it does not represent a naturally occurring protein, it displays strong antibacterial and candidacidal activities; although its antiviral activity remained unknown until 2014, when Yin and co-workers [94] described it. These authors reported that the second putative antimicrobial domain of lactoferrin was responsible for

its antimicrobial activity. This domain interacts with glycerol's head groups in multilamellar vesicles following a two-step model; the first 11 residues of the peptide adopt an amphipathic α-helical conformation, while the C-terminus of the molecule remains relatively unstructured [91], and this conformation causes membrane instability and is responsible for the antimicrobial activity of the compound.

2.2.2.4. Lactogenin and glycolactin. Lactogenin (17 kDa, P59761) was isolated from bovine milk [95] and displays limited ribonuclease activity on yeast transfer RNA (tRNA), as well as cleaving polyC. Other activities of this protein include inhibiting cell-free translation in a rabbit reticulocyte lysate, inhibiting the HIV-1 integrase (Fig. 1.4) and moderately inhibiting the reverse transcriptase (Fig. 1.3) [96], and weakly inhibiting HIV-1 protease (Fig. 1.5) [87]. Lactogenin, under the name angiogenin-1, was characterized as a glycyrrhizin-binding protein that can be phosphorylated by C-kinase, and this fact helps understand the *in vivo* mechanism of action of the protein [76].

Ye and Ng described in 2000 [97] a milk glycoprotein with an apparent molecular weight of 64 kDa capable of inhibiting the hemagglutinating activity of soybean agglutinin 120. This protein, as is the case for lactogenin and glycolactin (P59760), displayed moderate RNase activity on yeast tRNA, with a pH optimum of 7.5, and could also inhibit cell-free translation in a rabbit reticulocyte lysate. This mechanism of action made it likely that glycolactin would display antiviral activity, and this activity was demonstrated by Wang and co-workers in 2000 [81]; they found that both glycolactin and its succinyl derivatives could inhibit HIV-1 reverse transcriptase (Fig. 1.3). The following year [87] it was finally confirmed that glycolactin is also a strong inhibitor of both the HIV-1 protease (Fig. 1.5) and integrase (Fig. 1.4), thus inhibiting viral cycle of this virus.

2.2.2.5. β-lactoglobulin. β-lactoglobulin (P02754) is an old friend of biochemistry students, because it was the protein traditionally used to study dicarboxylic and basic amino acids [98], and it was even used in monolayer formation in those early years [99]. As reviewed by Pellegrini and co-workers in 2001 [100], this anionic protein [18.3 kDa, Isoelectric point (pI) 4.8] is most abundant in ruminants, but absent in humans, although its tertiary structure is homologous to the human serum retinol binding protein [101]. The β-lactoglobulin molecule contains four bactericidal domains, with activity mainly against Gram positive bacteria [100]. The amino acid sequence of β-lactoglobulin spans 162 residues (shown below), with the four bactericidal domains displayed in bold:

LIVTQTMKGL.DIQK**VAGTWY**.SLAM**AASDIS**.**LLDAQSAPLR**.VYVEE LKPTP.EGDLEILLQK.WENGECAQKK.IIAEKTK**IPA.VKF**IDALNEN. K**VLVLDTDYK**.KYLLFCMENS.AEPEQSLACQ.CLVRTPEVDD.EALEKFD KAL.KALPMHIRLS.FNPTQLEEQC.HI.

The bactericidal peptides can be released, by proteolytic digestion, by endopeptidases present in the mammalian gastrointestinal tract. Simple chemical modifications in the β-lactoglobulin molecule can produce peptides with antiviral activity, one such modification is the formation of 3-hydroxyphthalic anhydride, which produces a compound that blocks the cellular entry of HIV [102] and herpesviruses [103,104] via the CD4 cell receptor (Fig. 1.1). In addition, methylated derivatives of β-lactoglobulin interfere with the ability of either human cytomegalovirus (HCMV) [69] or human influenza virus A subtypes H3N2 [105] and H1N1 [106] to generate viral progeny. Furthermore, these modified antivirals have been assessed as safe for therapeutic use in humans, with no associated side effects to the patient [107,108].

2.2.2.6. Casein. Casein is the main protein in mammalian milk (it constitutes *ca* 80% of the protein content in bovine milk) and contains several proteinaceous varieties, such as 3 phosphoproteins, αS1-, αS2-, and β-casein. Another component is the phosphoglycoprotein κ-casein, that uniquely exhibits 3 O-glycosylation sites (Thr_{131}, Thr_{133}, and Thr_{135}) [109], thus the polypeptide moiety is attached to a glycan structure that changes during the secretion process [109,110]. The glycosylation pattern of the κ-casein present in colostrum, after parturition, contains one neutral oligosaccharide alditol and three acidic oligosaccharide alditols (one tri, one tetra and one pentasaccharidito l). Saito a colleagues [110] identified the chemical structures of these glycans, by gas-liquid chromatography-mass spectrometry and by anomer analysis with 13C nuclear magnetic resonance, and found galactosyl-β-1-3[galactosyl-β-1-4-N-acetylglucosaminyl-β-1-6-]-N-acetylgalactosaminitol, N-acetylneuraminyl-α-2-3-galactosyl-b-1-3-[galactosyl-β-1-4-N-acetylglucosaminyl-β-1-6-]-N-acetylgalactosaminitol, N-acetylneuraminyl-α-2-3-galactosyl-β-1-3-N-acetylgalactosaminitol, and N-acetylneuraminyl-α-2-3-galactosyl-β-1-3-[N-acetylneuraminyl-α-2-6-]-N-acetylgalactosaminitol. It has been reported that both human (P07498) and bovine (P02668) κ-caseins have protective properties against human rotavirus (HRV) infection, a major etiologic agent of severe infantile gastroenteritis. Although Isa and co-workers carried out the pioneering work in 2006 [111], it was Inagaki and colleagues, in 2014 [109], who described the role of glycans and sialic acids (such as N-acetylneuraminic acid) in the anti-rotavirus activity of κ-caseins. The anti-HRV activity of κ-casein was found to be due to its *o*-linked glycans, as deglycosylated κ-casein lacks antiviral properties [109].

2.2.3. Modifications of milk proteins to increase their antiviral activity

As indicated above, whey proteins display activity against a variety of viruses, such as Semliki forest virus, adenovirus, HCMV, HIV, enterovirus, rotavirus, Sindbis virus, echovirus, papillomavirus, hantavirus, HSV 1 and 2, respiratory syncytial virus, influenza virus H1N1, avian influenza virus H5N1, poliovirus, HBV, and HCV [86].

Early reports showed that simple modifications of the lysozyme molecule by irradiation generated derivatives with different biochemical properties [112]; the authors, however, did not describe the biological activities of the modified compounds. A short while later, another lysozyme derivative was obtained, this time by chemically modification of the protein's arginine residues with phenylglyoxal [113]. That was followed by lysozyme derivatives with modified tyrosine or tryptophan residues (i.e. tyrosines 20 and 23 were nitrated to nitrotyrosins or these reduced to aminotyrosins and the 6 tryptophans in turn were modified by reaction with 2-nitrophenyl sulfenyl chloride [114,115]), and in 1971 new lysozyme derivatives were generated by modifying its lysine residues by either guanidination, acetylation, succinylation, or maleylation [116,117]. Other simple lysozyme modifications include addition of fluorescein-isothiocyanate [118], chemical conversion of aspartic acid 52, a catalytic residue in hen egg-white lysozyme, to homoserine [119], ozone oxidation of $tryptophan_{62}$ to N′-formylkynurenine [120,121], selective modification of the aspartic acid at position101 in lysozyme by carbodiimide reaction [122], and modification of the enzyme's catalytic groups with ethylenimine [123]. All this research into lysozyme derivatives finally led into more radical and permanent modifications of the protein, such as the ADP-ribosylation of lysozyme described by Skórko and Kur [124]. Some years later, it was found that modification with polyanionic substances, such as heparin or sulfated polysaccharides [125,126], generated derivatives capable of inhibiting HIV-1 replication. However, it was an unexpected finding, supported by previous works on antiviral peptides generated by lysozyme digestion [127] or LALBA [128], that shed new light into antiviral proteins. The work by Oevermann and coworkers [68] confirmed that protein fragments obtained from naturally-occurring proteins

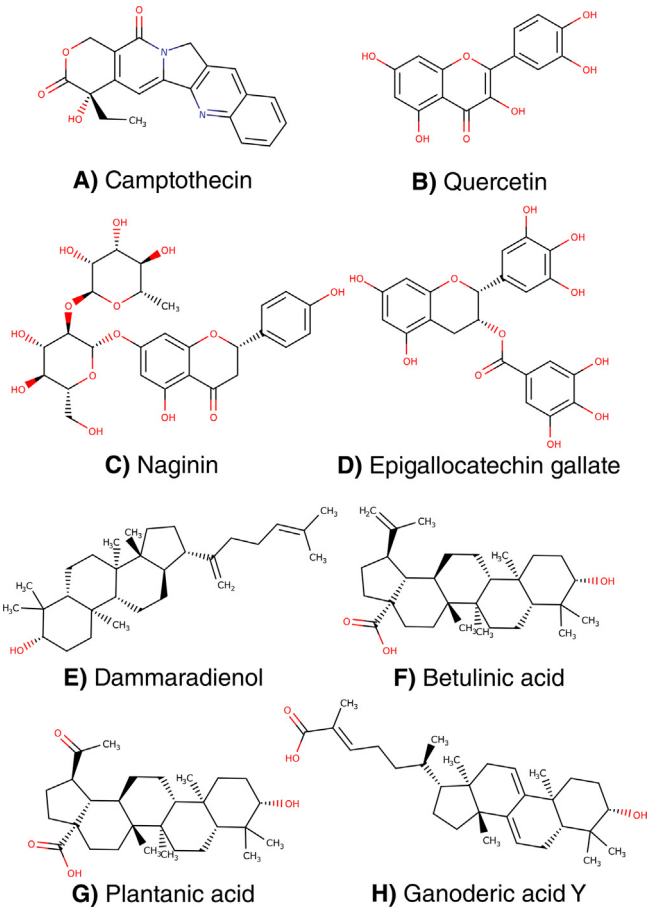

Fig. 5. Chemical structure of several antiviral compounds produced by plants. A. Camptothecin (CID: 24360). B. Quercetin (CID: 5280343). C. Naringin (CID: 4441). D. Epigallocatechin gallate (CID: 65064). E. Dammaradienol (CID: 13893946). F. Betulinic acid (CID: 64971). G. Plantanic acid (CID: 64980). H. Ganoderic acid Y (CID: 57397445). The chemical structures were obtained from the PubChem Database (https://pubchem.ncbi.nlm.nih.gov/) and represented using the MarvinSketch 16.5.23.0 software (ChemAxon Ltd, Budapest, Hungary).

after chemical modification exhibited improved antiviral activities. In particular, tryptic peptides, obtained from 3-hydroxyphthalic anhydride-modified bovine serum albumin, LALBA, β-lactoglobulin, and chicken lysozyme [68,102,104], were successfully tested on herpes virus-infected Vero cells. The results obtained were quite promising, but unfortunately some of the peptides had cytotoxic effects on the Vero cells.

3. Antivirals from plants

Plants have long represented a good source of antimicrobial molecules and, in a manner mirroring the pioneer work of Waksman [129] on streptomycin (CID: 19649) production by *Streptomyces* species, plants were found to be a source of antibiotics, effective against Gram positive and Gram negative bacteria, as well as fungi [130]. This clearly indicated that plants could also be a good source of antivirals. Consequently, Fischer and coworkers reported in 1954 [131] that some vegetable tannins displayed virucidal and antiviral effects, and Ragetli and Weintraub [132] described that a proteinaceous compound from *Dianthus caryophyllus* could drastically inhibit the replication cycle of tobacco mosaic virus (TMV) infecting *Nicotiana glutinosa*. Albano and Donato reported similar findings for human viruses, such as polioviruses and ECHO (enteric cytopathic human orphan) viruses [133]. Sela and Applebaum [134] are among the pioneers in this field, they described the production of antiviral substances by plants such as *Melissa officinalis*, that produces a cytotoxic quinoline alkaloid [135], or *Camptotheca acuminate*, that contains camptothecin (Fig. 5A, CID: 24360), a substance capable of inhibiting DNA topoisomerase I [136,137], in its bark and stem. Other antivirals include the tannin and non-tannin polyphenols from *M. officinalis* [138,139], an alkaloid fraction from *Narcissus* [140], and the hymnemic acids capable of viral modulation [141]. Krmpotic and colleagues reported in 1972 [142] the isolation of cryptopleurine (CID: 92765), an antiviral alkaloid from *Boehmeria cylindrica* (Urticaceae), highly active against DNA animal viruses, such as HSV-1. Although this compound displayed no activity against some of the RNA viruses tested, such as Coxsackie B-5 and polio type I viruses. Van den Berghe and co-workers reviewed, in 1978 [143], the published data on one hundred plant extracts, belonging to 73 genera and 43 families, and found that only eight of these extracts exhibited a significant antiviral activity.

3.1. Flavonoids

Flavonoids are an important group of plant-derived secondary metabolites with not only antiviral activity (with more than 5000 compounds described [144]), but also effective against cardiovascular diseases, ulcers, inflammation, osteoporosis, diarrhea, and arthritis. Flavonoids are, by far, the most abundant polyphenols in the human diet; usually found as glycosides, and sometimes as acylglycosides, in fruit and vegetables. Naturally occurring flavonoids, such as quercetin (Fig. 5B, CID: 5280343), naringin (Fig. 5C, CID: 4441), hesperetin (CID: 72281), and catechin (CID: 1203), have been tested against several animal viruses, including HSV-1, polio-virus type 1, parainfluenza virus type 3, and respiratory syncytial virus [145], with different effects displayed depending on the flavonoid compound and the target virus, suggesting a certain specificity of the antiviral mechanism of action. The antiviral activity of flavonoids was soon found to include adenoviruses, Rous sarcoma, Sindbis, pseudorabies [146,147], severe acute respiratory syndrome coronavirus (SARS-CoV) [148], and influenza A virus H1N1 [149]. Epigallocatechin gallate (CID: 65064), also known as epigallocatechin-3-gallate (Fig. 5D), is the most abundant catechin in tea, and has recently been shown to exhibit broad activity against a variety of unrelated viruses; this includes both DNA and RNA viruses, such as HCV, influenza, vaccinia, adenovirus, reovirus, and vesicular stomatitis virus [150]. Therefore, this opens the possibility of developing small antiviral molecules that are active against the two largest groups of pathogenic human viruses, those that bind to glycosaminoglycans (i.e. HSV) and those that bind to sialoglycans (i.e. influenza viruses). The mechanism of action of these compounds is based on their inhibition of cellular receptor kinases, such as the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3-K), the serine/threonine-specific protein kinase (Akt) and the mitogen activated protein kinases (MAPKs), thus interfering with cellular signal transduction pathways.

3.2. Triterpenes and triterpenoids

Triterpenes (consisting of six isoprene units, with squalene as prototype) are abundant plant compounds (ca. 30,000 according to Dzubak et al. [151] and Malinowska et al. [152]) that play a variety of roles in their cellular metabolism, they are sterol precursor and can display antiviral activity. Nine triterpenes with antiviral activity against HSV1 and HSV2, dammaradienol (Fig. 5E, CID: 13893946), dammarenediol-II (CID: 10895555), hydroxydammarenone-I (CID: 44584517), ursonic acid (CID: 9890209), hydroxyhopanone (CID: 21582894), dammarenolic acid (CID: 22215841), shoreic acid (CID: 12315515), eichlerianic acid (CID: 12315516), and hydroxyoleanonic lactone (CID: 44584523),

were purified by Poehland and co-workers in 1987 [153]. Betulinic acid (CID: 64971) is a pentacyclic triterpenoid (Fig. 5F), isolated from bark of the white birch tree (*Betula alba* var. *pubescens*), which has antiretroviral and anticancer activities. This compound inhibits eukaryotic topoisomerase I [154], and its derivative dihydro betulinic acid displays the strongest biological activity. Betulinic acid has been reported to cause apoptosis by the MAP kinase pathway [155]; this includes caspase activation, mitochondrial membrane alterations and DNA fragmentation [156,157]. Platanic acid (Fig. 5G, CID: 64980), isolated from the leaves of the trumpet satinash (*Syzygium claviflorum*) by Fujioka and colleagues in 1994 [158], is another pentacyclic triterpenoid with antiretroviral activity and similar mode of action. In addition, neem leaves (*Azadirachta indica* A. Juss) contain triterpenoids that exhibit activity against group B coxsackieviruses [159], and seeds and stems of *Caesalpinia minax* are good sources for rare cassane furanoditerpenoids and a friedelane triterpenoid [160], with activity against human parainfluenza virus type 3.

Virus reactivation, even after long periods of latency in the nuclei, is very problematic in medicine and a worldwide cause of human morbidity and mortality. The activation of Epstein-Barr virus (EBV) is associated to diseases such as infectious mononucleosis, or "glandular fever", as well as cancers such as Hodgkin's lymphoma, Burkitt's lymphoma, gastric cancer, nasopharyngeal carcinoma. In fact, it is estimated that close to a quarter of a million cancer cases worldwide, every year, are due to reactivation of this DNA virus. Hence preventing EBV virus reactivation could result in reducing, or even eliminating, both infectious mononucleosis and the above cancers. In this regard, compounds such as 3-epicabraleahydroxylactone (CID: 12080691), and other triterpenoids isolated from camellia oil, have been shown to display strong activity against this virus [161]. More recently, flavaglines and triterpenoids from the leaves of *Aglaia forbesii*, have been used as antivirals, also with promising results [162].

In 2012, Osorio and co-workers reported the isolation of nine new olean-18-ene triterpenes from *Cassine xylocarpa* and *Maytenus jelskii*, and some these compounds were already know to be active against HIV, the etiological agent of AIDS (acquired immune deficiency syndrome) in humans, by targeting the transcription factors NF-*k*B (nuclear factor kappa-light-chain-enhancer of activated B cells) and Sp1 (a zinc finger protein that binds GC-rich motifs present in many promoters). This anti-HIV activity is very important as, despite the fact that HIV was characterized in the 1980s, this disease cannot yet be cured and remains a serious public health problem [163]. Chang and colleagues, also in 2012, reported that *Euphorbia neriifolia* produces 22 triterpenoids with stronger activity against human coronavirus than actinomycin D (CID: 2019) [164]. Argan oil, from *Argania spinose,* in addition to containing oleic acid (44%, CID: 445639), α-linolenic acid (30%, CID: 5280934), palmitic acid (12%, CID: 985), stearidonic acid (6%, CID: 5312508), linoleic acid (5%, CID: 5280450), and myristic acid (3%, CID: 11005), also contains triterpenoidic derivatives that could potentially be used as antivirals; in particular, this is the case for arganine C (CID: 21603545) that represents a lead compound for the development of new antivirals [165].

Human enterovirus 71 infection can constitute a serious health threat in children under 7 years of age, causing worldwide neurological and clinical disorders that affect the children's feet, hands and mouths [166]. But recently [167], two novel *Ganoderma lucidum* triterpenoids, lanosta-7,9(11),24-trien-3-one,15;26-dihy droxy and ganoderic acid Y (GLTB, CID: 57397445, Fig. 5H), have been successful against EV71 infection; and these triterpenoids did not display cytotoxicity in humans. In addition, Alvarez and co-workers [168] found that lupene-derived pentacyclic triterpenoids, from *Bursera simaruba,* are capable of inhibiting HSV-1 and HSV-2 replication. Furthermore, Kvasnica and colleagues [169] reported the synthesis and medical significance of pentacyclic triterpenoids, with nitrogen- and sulfur-containing heterocycles, that displayed antitumor and antiviral activities, and concluded: "*The incorporation of heterocyclic motifs into triterpenes sometimes brings a new mechanism of action, increased activity and better pharmacological properties, which makes the area of heterocyclic triterpenes a hot topic in the chemistry of active natural compounds*".

The old classical method of extracting compounds from plants with organic acids is being slowly substituted by *in vitro* plant callus cultivation and biotransformation, to originate a microbial-mediated variety of triterpenoids with more appropriate antiviral properties [152]. This also permits to optimize the yield and quality and produce standardized target compounds [170]. This new technology has been successfully applied in the biotransformation of oleanolic acid by *Rhizomucor miehei*, ursolic acid by *Syncephalastrum racemosum*, lupeol by *Mucor rouxii* and *Aspergillus ochraceus*, betulin by *Aspergillus oryzae* and *Aspergillus foetidus*, and, finally, betulinic acid by *Mycelia sterilia* and *Penicillium citreonigrum* [152].

3.3. Interferon-like proteins

Plants often express viral resistance following the interaction of a pathogenic compound (avirulent product gene, *avr*) with a cellular receptor-like plant protein or resistance gene product (R), and this triggers a cascade of downstream-transduced events that generates a resistance phenotype. According to Levy and colleagues (2004) [171], this represents a consensus model based on Flor's gene-for-gene theory [172,173]. A typical example of R is the protein N of *Nicotiana tabacum*, introduced into tobacco from *N. glutinosa*, and the *avr* counterpart in TMV is the helicase domain of the viral 126-kD protein, that is part of the virus replicase complex. Sela and Applebaum [134] suggested that plants can produce interferon-like proteins that, as it is the case for interferon in vertebrates, could generate an antiviral state [174]. Indeed, human interferons can reversibly protect plants from viral infection, in particularly they protect several *Nicotiana* species from TMV infection [175]. This antiviral factor was identified as a glycoprotein, induced by both TMV and poly(I,C), that generates a nucleotide similar to the 2′,5′-oligoisodenylate (see interferon section), which in turn confers the antiviral activity [176]. These findings indicate that plants and animals probably share a common virus resistance pathway. Indeed, Western blot analysis of crude protein extracts, from tobacco leaves infected with TMV, with polyclonal antibodies against human β-interferon identified two plant proteins, although the sequence of the peptides obtained displayed no significant homology to human beta-interferon [177]. Those two proteins were characterized the following year [178] as a β-1,3-glucanase and an isoform of PR-5. But the idea that plant proteins can display antiviral activity is gaining force every day, and confirms previous reports, such as that of Awasthi in 1981 [179], that found a small protein of 14–18 kDa, isolated from *Cuscuta reflexa,* with strong activity against a variety of viruses. Another such an example was provided by Ready and co-workers who, in 1984 [180] and 1986 [181], reported that both dodecandrin (CID: 71301031) and the pokeweed antiviral protein (two novel ribosome-inhibiting proteins purified from *Phytolacca dodecandra* and *Ph. Americana*, respectively) display strong antiviral activity.

In the foreseeable future, we can expect that complete bacterial operons will be routinely cloned and non-transgenically expressed, using the IL-60 construct derived from the geminivirus Tomato Yellow Leaf Curl Virus (TYLCV) as the platform, to produce antivirals or other metabolites [182]. This novel technology, with an expression rate close to 100%, eliminates the need to use selectable expression markers.

4. Antivirals from fungi

Early publications by Shope [183–185] reported that the fungus *Penicillium funiculosum*, isolated on Guam, produced a substance therapeutically active on mice infected with swine influenza virus. But production of the active principle was erratic and the fungus eventually lost this property. The author then claimed that *Penicillium funiculosum* produced a second substance, called "helenine", that was clinically active against Columbia SK encephalomyelitis virus. Shope suggested that helenine temporarily interfered with the virus neuroinvasiveness in infected mice, but neither the mechanism of action nor the chemical structure of the compound was unraveled at the time, although the author found that crude active preparations of helenine contained a large proportion of polysaccharide. Finally, Shope concluded: "*From the facts discussed thus far it is quite apparent that helenine would have no practical value in treating naturally occurring outbreaks of virus diseases resembling the experimental SK virus infections in mice*". Some years later it was reported that statolon [CAS: 11006-77-2], a complex anionic polysaccharide with a relatively high galacturonic acid (CID: 439215) content, produced by *Penicillium stoloniferum*, was involved in interferon production in animal cells [186], hence creating an antiviral state. Some years later, Fujii and colleagues [187] isolated an *O*-glycosylated mannan, from *Lentinus edodes*, that could suppress the growth of both Ehrlich and Sarcoma-180 tumors in mice. *Lentinus edodes* represents the first medicinal macrofungus to enter the realm of modern biotechnology. The therapeutical importance of this fungus is not limited to its antiviral properties, as it also useful in the treatment of diseases involving a weakened immune system, cancer, environmental allergies, fungal infection, and patients suffering from frequent flu and colds, bronchial inflammation, heart disease, hyperlipidemia (including high blood cholesterol), hypertension, infectious disease, diabetes, hepatitis, and it even plays a role in regulating urinary incontinence [188].

Dextran sulfate (CAS: 9011-18-1), a glucose homopolymer with a molecular mass between 7000 and 8000 Da that contains 17–20% of its sulfur as sulfate, inhibits the replication of not only the human immunodeficiency virus, but also of other retroviruses. It does this mainly by blocking the virus attachment to the host cell (Fig. 1.1) or its retrotranscriptase activity (Fig. 1.4), but it is effective against viruses at high doses, which are toxic to the patients. Other compounds have been tested for their antiviral activity, causing cells to slowly develop an antiviral state. This suggests that polysaccharides that humans cannot hydrolyze (such as 1,3-β-D-glucans) display an indirect mode of action to induce a cellular antiviral response, as suggested by Veiga-Crespo and Villa in 2010 [189]. These compounds include sulfated polysaccharides, such as curdlan (CID: 64689, a 1,3-β-D-glucan), and neutral polysaccharides, such as schizophyllan (CID: 24777) or scleroglucan (CAS: 39464-87-4) (β-1,3 beta-glucans with β-1,6 branching, produced by *Schyzophyllum commune* and *Sclerotium rolfsii,* respectively), as well as a 1,3-β-D-glucan from the sclerotia of *Pleurotus tuber-regium* [190], and sulfated polysaccharides from either *Agaricus brasiliensis* mycelia [191,192] or *Auricularia auricula* [193]. The enzymes that can hydrolyze these polysaccharides (i.e. 1,3-β-D-glucanases) are commonly found in fungi, bacteria and plants, but their distribution in the animal kingdom is rather limited, as they have only been found in the eggs and digestive tract of echinoderms and frogs [194]. Hence, 1,3-β-D-glucanases have not been found in primates, and no structural genes have been discovered in humans. This means that when a non-hydrolysable polysaccharide molecule is presented to the human immune system, it activates unspecific responses that include the generation of an antiviral state, sometimes mediated by an increase in γ-interferon [195].

Heparin is a highly sulfated glycosaminoglycan that displays anti HSV-1 activity by interfering with the binding of viral glycoproteins B and C to the proteoglycans on the cell surface (Fig. 1.1) [196]; but its blood thinning effect precludes its use in human medicine. This, in addition to the fact that heparin-like polysaccharides are extremely rare in fungi, limits the use of sulfated fungal polysaccharides as antivirals, as they appear to only have a collateral effect, except for the complex fungal polysaccharides chitosan and chitin. Chitosan (CID: 71853) has been known for years to have antiviral activity [197], although its detailed mechanism of action remains elusive. This antiviral action could be related to the fact that chitosan can reversibly disrupt cellular tight junctions, thus facilitating the access of antiviral drugs into the cells [198] and perhaps even antiviral proteins, such as interferons, defensins, or other antimicrobial peptides produced by the animals in response to viral infection. Additionally, mammals have trouble metabolically processing chitosan and chitin, as well as 1,3-β-D-glucans, and those compounds linger inside the body, hence it is likely that the organism deploys a variety of countermeasures (including antivirals) when encountering these complex polysaccharides. In fact, mammals express only two genes with chitinolytic activity, a chitotriosidase and a prototypic acidic chitinase, that can decrease the amount of circulating polysaccharide. These enzymes have been associated in humans with different syndromes, such as Gaucher disease, liposomal lipid storage, thalassemia, and even psychiatric disorders (for a full report on these enzymes in humans, see Veiga-Crespo and Villa [189]). Li and co-workers described in 2011 [199] a new application for chitosan-based materials in the prevention and control of influenza virus. They used chitosan functionalized with sialyllactose and found that it drastically inhibited viral adsorption (Fig. 1.1), as the chitosan-conjugate bound to the viral hemagglutinin with high affinity and prevented viral attachment to the host. Ciejka et al. recently synthesized new sulfonated derivatives of poly(allylamine hydrochloride) (CAS: 71550-12-4) and N-sulfonated chitosan and tested them against influenza A and B viruses [200]; the authors found that these novel molecules could inhibit influenza A and B virus assembly inside the host cell (Fig. 1.1).

Hyaluronic acid (CID: 24728612) is a non-sulfated glycosaminoglycan found in synovial fluid and eye vitreous humor, and common in connective tissue; but although it is commonly found in bacteria, it is rare in fungi (with the exception of *Cryptococcus*). Medical uses of hyaluronic acid include wound healing and mouth care, as well as the treatment of conditions such as joint pain, kerato-conjunctivitis, asthma, and gastritis. This compound, in addition, displays antiviral activity against a variety of DNA and RNA viruses [201]. But, despite its positive effects, the fact that hyaluronic acid could enhance blood-brain barrier permeability, either transcellularly, paracellularly or through infected phagocytes [202], considerably limits its medical uses.

5. Antivirals from bacteria

It has long been known that some bacteria can produce antiviral substances, such as the macrolide antibiotic borrelidin (CID: 6436801), produced by *Sptreptomyces* sp [203] (recently rediscovered and named *Streptomyces heilongjiangensis* [204]). Other antivirals include ehrlichin (synthesized by *Streptomyces lavendulae*) [129], abikoviromycin (CID: 6450263, produced by several species of *Streptomyces*) [205], violarin (CID: 44256909, from *Actinomyces violaceous*) [206], myxoviromycin (CID: 160703, a compound produced by *Streptomyces* sp, that targets orthomyxoviruses)

[207,208], virocidin (CID: 9989534) [209], niromycins (CAS: 101997-22-2 and CAS: 101997-20-0, from actinomycetes) [210], extracts from propionibacteria [211], and vivomycin (CID: 3037981, produced by *Streptomyces* C2989) [212,213].

Feingold and co-workers [214] suggested in 1976 that *Brucella abortus* produced a compound, also present in *E. coli*, that could induce an antiviral state via interferon production, but they were unable to characterize the compound. Another early publication, by Glaser et al. [215], reported that rat spleen cells cultured with bacterial lipopolysaccharide (LPS) exerted cytotoxic activity against Gross virus (a retrovirus that causes lymphoma in rats, whose counterpart in humans is human T cell lymphotropic virus I, HTLV-I). It is well known that lipopolysaccharide, the major outer membrane component in Gram-negative bacteria, is one of the most potent activators of the innate immune system, which constitutes the host defense against invading pathogens, including viruses. Helfgott and colleagues [216] found in 1987 that LPS enhances the expression and secretion of beta 2 interferon by human fibroblasts, thus contributing to the creation of an antiviral state. This enhanced expression is mediated through the antiviral transcription factors STAT-1 and NF-kappa B. The former is a member of the STAT protein, involved in gene upregulation through signaling by type I, type II, or type III interferon; while the latter is a protein complex that controls DNA transcription, cytokine production and cell survival. LPS can produce unexplained effects when supplemented to virus-infected cells. In addition, low LPS concentrations induce macrophage-binding to virally infected cells [217]. LPS can also exert peculiar effects, as reported by Canavese and colleagues in 2015 [218]. They found that LPS, in combination with vascular endothelial growth factor, causes a strong synergistic effect that protects from cerebral malaria, caused by *Plasmodium berghei*. In addition, it was recently reported [219] that cellular caspases become activated through oligomerization upon interaction with LPS, which leads to a variety of cellular effects that could involve generation of antiviral states.

Parts of the LPS molecule, such as the monophosphoryl derivative of lipid A (CID: 9877306), have been reported to have antiviral activity, this however appears to be due to its adjuvant activity when formulated in vaccines [220–222]. Indeed, Patil and colleagues confirmed in 2014 [223] the adjuvant activity of monophosphoryl-Lipid A as part of a pulmonary delivered influenza vaccine. However, we cannot ignore the fact that lipid A induces inflammation in the mammalian immune system [224,225], even in picomolar amounts, by triggering TLR4/MD activation; hence lipid A itself can be considered an endotoxin. Lipid A-derived molecules, however, can display interesting properties as antivirals. Accordingly, Ikeda and coworkers reported in 1990 [226] that chemically-synthesized lipid A analogs, containing acyl side chains between C8 and C15, had significant antiviral activity (orthopoxviruses), serum IFN-inducing activity and NK cell activation; in addition, the compounds with a C13 or C14 acyl side chain protected immunosuppressed mice against HSV1. Other bacterial components, including cell walls and even peptidoglycan in Gram positive bacteria (i.e. *Bacillus alcalophilus*), can activate macrophages and natural killer cells, creating an anti-poxviral state through increased α-interferon levels [227]. But this antiviral state is different from the one generated by LPS from different bacteria, as shown by Winters and colleagues in 1985 [228]. These authors treated mice by intraperitoneal inoculation of *Bordetella pertussis* lipopolysaccharide and found that this created resistance to rabies virus, encephalomyocarditis virus, Semliki Forest virus, and Herpes simplex virus, whereas the LPS from *E. coli, Vibrio cholerae, Salmonella typhimurium, and Salmonella minnesota* did not.

Highly sulfated K5 *E. coli* polysaccharide derivatives effectively inhibit the capacity of respiratory syncytial virus to infect humans [229]. LPS can cause, through Toll-like receptors, the induction of harmful immune and proinflammatory genes. These negative effects could be counteracted by a LPS-like molecule extracted from the cyanobacterium *Oscillatoria planktothrix* that, although not stimulatory *per se,* acts as a selective antagonist of the antiviral bacterial LPS [230]. Bacterial exopolysaccharides can induce cells to produce a variety of cytokines, thus each resulting in protection against different viruses. In this way, Maugeri and colleagues reported, in 2006 [231], a novel extracellular polysaccharide, from the thermotolerant *Bacillus licheniformis*, which induced an antiviral state through the production of IL-12, IFN-gamma, IFN-α, TNF-α, and IL-18, but not IL-4. This indicates that the antiviral effect of EPS-1 on PBMC is related to the particular cytokines induced.

Bacterial flagellin is a globular protein, with a molecular mass between 30 and 60 kDa (depending on the bacterial type), shaped as a hollow tube that constitutes the main component of the bacterial flagellum and is present in almost all mobile bacteria. Flagellin is also found in unexpected places inside bacteria, and it has been described as part of the *Bacillus subtilis* nucleoid [232]. Vijay-Kumar and colleagues found that systemic treatment of mice with purified flagellin did not induce the serological or histopathological side effects of LPS, even though it still protected the animals against challenges such as radiation or viral infections [233]. Although the mechanisms responsible for these protective roles are not clear, they could be related to the affinity that flagellin has towards DNA.

Apart from the bacterial compounds capable of eliciting a virucidal response reviewed, there are also a variety of bacterial virulence factors that can cause host tissue pathology, stimulating the mammalian cells to produce a diverse array of cytokines (that can result in the production of an antiviral state). These factors were classified by Henderson and co-workers in 1996 [234] into four families: adhesins, aggressins, impedins, and invasins; these substances are not included here because their antiviral action has not been confirmed.

6. Antiviral peptides (AVP) and antiviral enzybiotics (AVEB)

Since viruses replicate either in the cytoplasm or nuclei of eukaryotic cells (Fig. 1.4a and 1.4b), AVPs and AVEBs are the logical approach to control the diseases they cause. AVPs are short peptides that, once taken up by the eukaryotic cells, interfere with the replicative cycle of the virus. On the other hand, AVEBs are enzymes (i.e. proteases) that could extracellularly digest, either totally or partially, the viral capsid; this would render the naked viral nucleic acids susceptible to degradation by environmental DNases [235]. An alternative to this, as for example the stimulation of an antiviral state within the host cell through the production of interferon, has already been considered above. In synopsis, the use of enzybiotics in combination with synthetic antiviral drugs appears to have the best chance of success, but early treatment of viral infections can favor secondary bacterial infections, as it has been repeatedly reported for influenza viruses and *Streptococcus pneumoniae* or *Haemophilus influenzae* [236] or in anti-HCV treated patients [237].

6.1. AVP

Feglymycin (CID: 16130418) is an antimicrobial peptide (AMP) isolated from *Streptomyces* sp. DSM 11171 and capable of inhibiting not only HIV entry into the cell (Fig. 1.1) by targeting the viral envelope protein gp120 [238]. Melittin (CID: 16133648) is an amphipathic α-helical AMP isolated from the European honeybee (*Apis melifera*) venom, its mechanism of action involves blocking the virus-cell fusion (Fig. 1.1) [239]. Defensins are short cationic non-glycosylated peptides originally found in rabbit and human

neutrophils, but this term can be extended to similar AMPs from plants and insects. Defensins exhibit a broad spectrum of action against bacteria, fungi and viruses. The antiviral properties of many defensins are related to their lectin-like properties [240]. For instance, it was recently discovered that human α-defensin 1 (HNP-1, CID: 16130476, P59665) inhibits multiple steps in the entry and fusion of HIV-1 (Fig. 1.1 and 1.2) [241] *in vitro*. In addition, several defensins (NHP-1 and human α-defensin 5, HD5, Q01523) can inhibit human papillomavirus (HPV) infection, by blocking virion release from endocytic vesicles (Fig. 1.3). They are also active against human adenovirus (AdV), by stabilizing viral capsid proteins and preventing capsid disassembly after cell binding (Fig. 1.3) [242]. θ-Defensins are a family of AMPs, related to α-defensin, found in some non-human primates. These AMPs display improved antiviral activities and can protect cells from infection by enveloped viruses, including HIV-1, HSV-1 and HSV-2, by inhibiting viral adhesion and entry [240]. The egg white in eggs from ancient Chelonioidea, such as the marine turtle *Caretta caretta,* was reported to contain a small cationic protein (TEWP, P0CAP0) with 36 amino acid residues, of which six are half-cysteines, with strong antiviral activity against both rhabdoviruses (i.e. vesicular stomatitis virus, VSV) and the emergent human pathogen Chandipura virus, although it also exhibited activity against Gram-negative bacteria [243].

6.2. AVEB

Ribotoxins are potential candidates to be included in the group of "enzybiotics against animal viruses". Restrictocin (ribonuclease mitogillin, P67876) is one of the first ribotoxins described, it is produced by *Aspergillus restrictus* and can inactivate ribosomes [244]. Restrictocin belongs to the barnase superfamily [245], which constitutes a large group of RNA-degrading enzymes, it is 149–150 amino acids long (i.e. RNase T1), and it is produced by a variety of fungi [246,247]. Several bacteria (i.e. *Shigella dysenteriae, S. boydii, S. sonnei,* and *E. coli* STEC) produce a ribotoxin, the Shiga toxin, that inhibits the translation apparatus in eukaryotic cells, and also displays a potent antiviral activity (i.e. suppression of bovine Leukemia virus-related spontaneous lymphocyte proliferation [248]). All ribotoxins preferentially enter virus-infected cells (although no cellular receptors have yet been identified) and cleave a unique phosphodiester bond located in the large rRNA gene, known as the *sarcin-ricin loop*, which leads to inhibition of protein synthesis and apoptosis [246]. In fact, ribotoxins first produce a 2′,3′-cyclic phosphate intermediate, via a transphosphorylation reaction [249], and consequently the intermediate is hydrolyzed to the corresponding 3′-phosphate [250]. Novel ribotoxin-derived antiviral immunotoxins are currently being developed [251] that lack detrimental side effects, generated due to the binding ability of ribotoxins to IgE antibodies. In 2008, Herrero-Galán and co-workers [252] reported the characterization of hirsutellin A (P78696), a ribotoxin spanning 130 amino acids produced by the mite fungal pathogen *Hirsutella thompsonii,* that has insecticidal and antiviral activities [253].

7. Synthetic antivirals

7.1. Small interfering RNA (siRNA)

Small RNAs from plants could soon have applications in pharmacology as antivirals. It is well known that, in plants, short interfering RNAs (siRNAs) confer intracellular antiviral immunity, thus serving as a natural antiviral defense mechanism. Elbashir and co-workers [254] showed that transfection of a 21-nucleotide long small interfering RNA (siRNA) into mammalian cells resulted in the degradation of the target mRNA, hence silencing gene expression

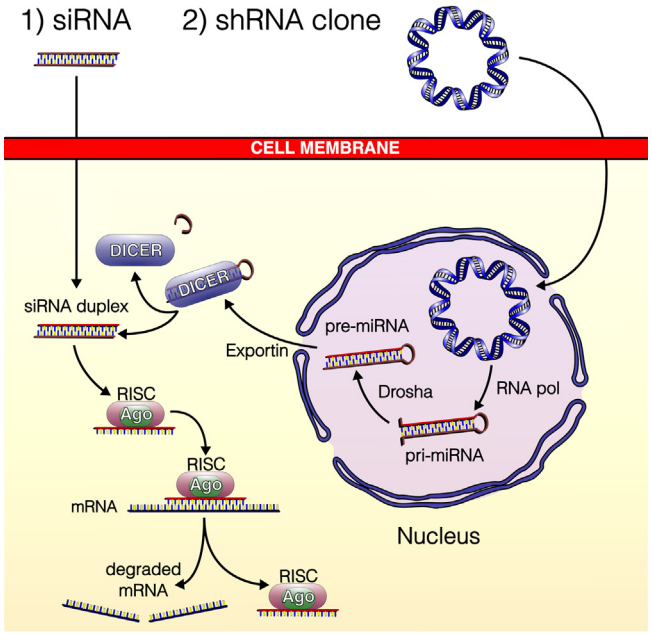

Fig. 6. Schematic representation of posttranscriptional gene silencing mediated by short interference RNA (siRNA) molecules and triggered by dsRNA. 1) siRNA introduced into the cell; 2) siRNA originated by transfecting the cell with a plasmid encoding a shRNA. The common point of all gene silencing pathways includes the formation of the RNA-induced silencing complex (RISC), 5′-phosphorylated siRNAs plus an endogenous Argonaute protein (Ago). The siRNA originating from the second approach, must be previously hydrolyzed by cellular RNAases III (Dicer and Drosha). Argonaute proteins bind different classes of small non-coding RNAs, including microRNAs (miRNAs), small interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs), and the RNAs guide the Argonaute proteins, through base pairing, to their specific sites for mRNA cleavage.

(Fig. 6). A year later, Gitlin and colleagues [255] reported that siRNAs elicited specific intracellular antiviral resistance in human cells; which suggested a novel therapeutic strategy against human viruses, as opposed to the traditional approach of using chemical antivirals. So, the following year Kapadia et al. [256] reported that short interfering RNAs could interfere with the RNA replication of HCV (Fig. 1.4). In 2005, Wu and co-workers [257] showed that infection by severe acute respiratory syndrome (SARS), caused by the newly discovered coronavirus SARS-CoV, could be controlled by this approach. In addition, Lecellier et al. designed 21- to 24-nucleotide long RNAs capable of controlling retroviral infection [258]. Therefore, the RNA silencing and RNA interference approaches, carried out by small RNAs such as small interfering RNAs (siRNA) and microRNAs (miRNA), appeared to successfully control viral infections [259,260]. Nevertheless, and despite all the expectations created in the scientific world, only a single drug, Fomivirsen (CID: 71587550, 5′-GCG TTT GCT CTT CTT CTT GCG-3′) has, thus far, been approved by the Food and Drug Administration; the approval is to treat cytomegalovirus-induced eye infections in acquired immunodeficiency syndrome patients [260]. The work of Shapiro and colleagues in 2014 [261], on siRNA interfering response, represented a step forward. The authors clearly demonstrated that Drosha, one of the two cellular RNAses III (Dicer and Drosha; Fig. 6), represented '*a unique and conserved arm of the cellular defenses used to combat virus infection*'. Field application of a combination of short interfering RNAs and adenoviruses expressing porcine α and γ interferon, appears to work well controlling the foot and mouth disease virus (FMDV) in swine [262]. In summary, the following RNA viruses could be inhibited using this new antiviral strategy: human respiratory syncytial virus (HRSV), human parainfluenza virus (HPIV), Dengue virus, HCV, polio-

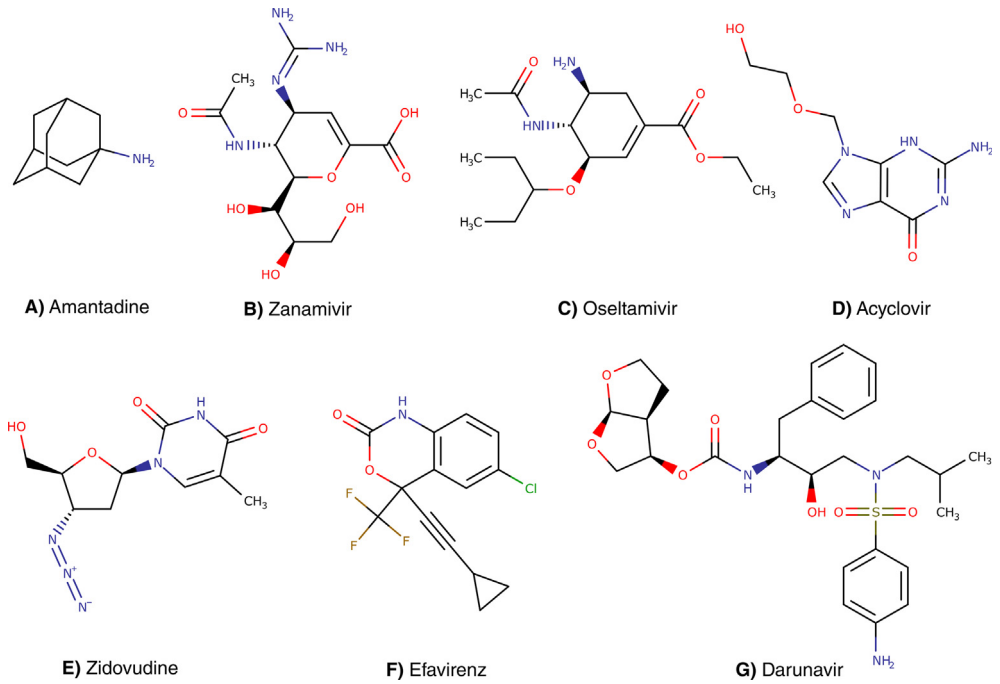

Fig. 7. Chemical structure of several synthetic antiviral compounds. A. Amantadine (CID: 2130). B. Zanamivir (CID: 60855). C. Oseltamivir (CID: 65028). D. Acylovir (CID: 2022). E. Zidovudine (CID: 35370). F. Efavirenz (CID: 64139). G. Darunavir (CID: 213039). Chemical structures were obtained from the PubChem Database (https://pubchem.ncbi.nlm.nih.gov/) and drawn using MarvinSketch 16.5.23.0 software (ChemAxon Ltd, Budapest, Hungary).

viruses, enterovirus 71 (EV71), Semliki forest virus (SFV), Ross river virus (RRV), feline herpesvirus (FHV), respiratory syncytial virus (RSV), porcine endogenous retrovirus (PERV), foot and mouth disease virus (FMDV), human hepatitis delta virus (HDV), SARS-CoV, human coronavirus NL63 (HCo-NL63), human rhinovirus (HRV-16), and avian influenza A virus. In addition, a number of DNA-containing viruses have been controlled using this approach, these include: human papillomavirus 16 (HPV-16), HBV, murine gammaherpesvirus 68 (MHV-68), HSV-1, John Cunningham virus (JCV), EBV, and HCMV.

7.2. Synthetic inhibitors of viral membrane fusion

Amantadine (1-Adamantanamine hydrochloride, CID: 2130, Fig. 7A) is a M2 proton channel blocker successfully used to treat influenza A virus infection [263]; it was discovered by Davies and coworkers in 1964 [264]. Amantadine is not a virucidal drug, and its antiviral effect is produced by inhibition of viral penetration (Fig. 1.1–1.3) [264,265]; the drug inhibits the M2 proton channel, hence the M1 protein cannot dissociate from the ribonucleoprotein complexes by acidification (Fig. 1.3) [266,267]. A similar mechanism of action was described for Rimantadine (α-methyl-l-adaman tanemethylamine hydrochloride, CID: 5071), an analog of Amantadine [266]. Arbidol [Umifenovir, ethyl-6-bromo-4-[(dimethyla mino)methyl]-5-hydroxy-1-methyl-2-[(phenylthio)methyl]-indol e-3-carboxylate hydrochloride monohydrate, CID: 131411] is a synthetic drug, developed in Russia in 1990 [268], with a broad spectrum of action. It is used to treat influenza virus (A, B and C) and, *in vitro,* it can suppress infection by Ebola virus, Tacaribe arenavirus, human herpes virus 8 (HHV-8), HCV, adenovirus, parainfluenza type 5, rhinovirus type 14, poliovirus, avian coronavirus, infectious bronchitis virus, Marek disease virus, HBV, and HCV [268,269]. Arbidol can also inhibit the replication of multiple virus families of medical importance worldwide, by blocking the virus-mediated fusion with the target cell membrane (Fig. 1.1) [268].

7.3. Synthetic neuraminidase inhibitors

Synthetic neuraminidase inhibitors (NAIs) were discovered in 1966 by Edmond and collaborators [270]. These compounds competitively inhibit viral neuraminidase (NA, sialidase, EC: 3.2.1.18), the enzyme present in influenza virus A and B that is responsible for the release of new virus particles from infected cells (Fig. 1.6). The authors synthetized several N-substituted oxamic acids which displayed NA inhibition and anti-influenza virus activity *in vitro*. However, these compounds lost their antiviral activity when injected into chicken embryonated eggs, presumably due to the oxamic acids being metabolized by the chicken embryo. DANA (Neu5Ac2en, 2,3-didehydro-2-deoxy-N-acetylneuraminic acid, CID: 65309) is a non-selective transition-state analog inhibitor of influenza NA [271] developed in 1974 [272]. DANA occupies the active site of the enzyme inhibiting its function, however, this compound has yet to demonstrate any beneficial effect in animal models of influenza infection [273]. Zanamivir (GG167, 4-guani dino-2,4-dideoxy-2,3-dehydro-N-acetylneuraminic acid, 4-guanidino Neu5Ac2en, CID: 60855, Fig. 7B) is a sialic acid analog designed based on the crystal structure of the influenza virus NA [273], and it was the first NAI approved for the prophylaxis and treatment of influenza infections [274]. Zanamivir shortens the duration of influenza symptoms and displays prophylactic effects in influenza patients, hence reducing the influenza-related complications [274]. This inhibitor is one million times more specific against viral NA than human NA [273], and it is administrated by inhalation (10 mg/dose) [275]. Oseltamivir [GS4104, ethyl(3R,4 R,5S)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate, CID: 65028, Fig. 7C] is the prodrug of GS4071 [(3R,4 R,5S)-4-acetamido-5-amino-3-(1-ethyl-propoxy)-1-cyclohexene-1 -carboxylic acid; CID: 449381], a potent inhibitor of viral NA [276,277]. Oseltamivir is administrated orally (75 mg/dose) [275] and, after absorption from the gastrointestinal tract, is enzymatically converted to GS4071, which subsequently penetrates into the bronchoalveolar lining fluid [277]. Peramivir [BCX-1812,

RWJ-270201, (1S,2S,3S,4R)-3-(1S)-1-acetylamino-2-hydroxycyclopentane carboxylic acid; CID: 154234] is a highly selective inhibitor of influenza A and B virus NAs; it was described by Babu and collaborators in 2000 [278]. Peramivir displays a greater inhibitory potency on viral NA, particularly on influenza B virus NA, than either zanamivir or oseltamivir [271]. Laninamivir [R-125489, (2R,3R,4S)-3-acetamido-2-[(1R,2R)-2,3-dihydroxy-1-methoxy-propyl]-4-guanidino-3,4-dihydro-2H-pyran-6-carboxylic acid; (2R,3R,4S)-3-acetamido-4-(diaminomethylideneamino)-2-[(1R,2R)-2,3-dihydroxy-1-methoxypropyl]-3,4-dihydro-2H-pyran-6-carboxylic acid; CID: 502272] is another NAI, developed in Japan in 2009, effective on oseltamivir-resistant influenza virus strains. Its esterified prodrug, CS-8958 (3-(*O*)-octanoyl R-125489; CID: 10412535), has a much longer half-life than laninamivir and zanamivir, as well as an improved efficiency [275].

7.4. Synthetic nucleoside analogs

Idoxuridine (IDU, 5-Iodo-2′-deoxyuridine; CID: 5905) is an iodinated analog of deoxyuridine (CID: 13712) synthetized by Pursoff in 1959 [279]. IDU is useful in the treatment of the ocular keratitis produced by HSV. This compound is transformed into triphosphate IDU and incorporated into DNA, inhibiting virus replication (Fig. 1.4). IDU is effective *in vitro* against most DNA viruses [280], these include HSV, HCMV and vaccinia virus [281]. Due to its toxicity [281], IDU is administered by dermal delivery, however, resistance to this compound has already been documented [280]. Acyclovir [Aciclovir, 9-(2-hydroxyethoxymethyl)guanine, CID: 2022, Fig. 7D] is one of the most prominent members of the acyclic nucleoside analog family [282], it is effective in the treatment of HSV-1 and 2, varicella-zoster, EBV, cytomegalovirus, and herpes B viruses [70,283,284]. This compound was synthesized in the 1970 s by Howard Schaeffer and Lilia Beauchamp [70]. Acyclovir is closely related to a natural DNA component (guanine deoxyriboside, CID: 187790), and, as acyclovir triphosphate (CID: 84000), it can inhibit DNA polymerase by competing with the natural deoxyguanosine triphosphate [283]. Although this effect is unspecific, the viral specificity of acyclovir arises from the fact that it is converted into its active form by a virus-specific thymidine kinase, which is not present in healthy cells [70]. Ribavirin (Virazole, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; CID: 37542) is an analog of guanosine (CID: 765), described in 1972 [285], with a broad-spectrum of antiviral action. It is used in the treatment of persistent infections by either HCV or respiratory syncytial viruses [276]. Ribavirin was reported to produce lethal mutations in the virus genome, resulting in reduced infectivity [286]. Zidovudine [ZDV, BW A509U, Azidothymidine (AZT), 3′-azido-3′-deoxythymidine, CID: 35370, Fig. 7E] is an analog of thymidine that acts as inhibitor/alternate substrate for the HIV-encoded reverse transcriptase, it limits viral replication (Fig. 1.4) [287,288]. ZDV is phosphorylated inside the cells after absorption, and its triphosphated form (CID: 72187) binds more strongly to viral reverse transcriptase than to the cellular DNA polymerase [276]. But its mechanism of action is virus-specific, since ZVD is effective against HIV but not HCMV [289]. ZDV was the first compound used to treat AIDS [290]. Didanosine (ddI, 2′,3′-dideoxyinosine; CID: 50599) and zalcitabine (ddC, 2′,3′-dideoxycytidine; CID: 24066) are two nucleoside analogs developed by Mitsuya and Broder in 1986 [291]. Both compounds are potent inhibitors of HIV replication, acting as chain-terminators of viral DNA, since this compound does not support nucleoside phosphodiester links at its 3′ end [291]. Other synthetic nucleoside analogs include stavudine (d4T, 2′,3′-didehydro-3′-deoxythymidine; CID: 18283), 2′,3′-didehydro-2′,3′-dideoxycytidine (d4C, DdeCyd, CID: 64683), lamivudine (3TC, 2′,3′-dideoxy-3′-thiacytidine; CID: 60825), emtricitabine [(-)-FTC, CID: 60877], tenofovir [(R)-9-(2-phosphonomethoxypropyl)adenine, CID: 464205; tenofovir disoproxil fumarate, CID: 6398764 (TDF, prodrug)], and adefovir [9-(2-phosphonyl methoxyethyl)adenine, CID: 60172] [284,288,292].

7.5. Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

Nevirapine (NVP, 11-cyclopropyl-4-methyl-5H-dipyrido[2,3-e:2′,3′-f][1,4]diazepin-6-one; CID: 4463) is a benzodiazepine NNRTI discovered in Boehringer-Ingelheim Pharmaceuticals by Hargrave and collaborators in 1991 [293]. This compound was the result of a random screening process for inhibitors of HIV-1 reverse transcriptase (RT), following a lead-optimization process. Nevirapine is a non-competitive inhibitor of HIV-1 RT, but not of HIV-2 RT or human DNA polymerase, and it was the first NNRTI compound to be approved by the FDA, in 1996. This compound allosterically binds to the tyrosine residues, at positions 181 and 188, in the p66 unit of HIV-1 RT. Efavirenz [EFV, DMP-266, (4S)-6-chloro-4-cyclopropylethynyl-4-trifluoromethyl-1,4-dihydro-2H-3,1-benzoxazin-2-one; CID: 64139, Fig. 7F] is another NNRTI of HIV-1 discovered in the Merck Research Laboratories [294]. EFV offers several advantages over NVP as a treatment for AIDS, although both of them are equally effective in suppressing the infection, these include a lower mortality and a lower incidence of AIDS-defining illness [292]. To avoid the appearance of resistance, EFV is used in conjunction with other antiretroviral therapies [295].

7.6. Protease inhibitors (PIs)

Synthetic PIs are antiviral drugs used in the treatment of HIV and HCV [282,295]. Viral proteases are essential for viral maturation (Fig. 1.5), for instance, in HIV and other retroviruses, Gag and Gag–Pol proteins must be cleaved to form the mature virion proteins [276]. Saquinavir (SQV, Ro 318959, CID: 441243), manufactured by Roche laboratories [290], is a peptidomimetic hydroxyethylamine inhibitor with high affinity for HIV protease [half maximal effective concentration (EC_{50}) of 2 nM]; it was the first PI approved by the FDA. Ritonavir (RTV, ABT-538, 1,3-thiazol-5-ylmethyl N-[(2S,3S,5S)-3-hydroxy-5-[[(2S)-3-methyl-2-[[methyl-[(2-propan-2-yl-1,3-thiazol-4-yl)methyl]carbamoyl]amino]butanoyl]amino]-1,6-diphenylhexan-2-yl]carbamate; CID: 392622) is a peptidomimetic PI developed by Abbott Laboratories in 1995 [296]. This compound is 100-fold more potent than SQV [290], however, it is highly inhibited by serum proteins [297]. RTV is used as a pharmacokinetic enhancer, since it boosts the effect of other antivirals by blocking their degradation by cytochrome P450 (the cytochrome P450 enzyme system is responsible for the metabolism of many drugs, including NNRTI and PI); hence, RTV is usually administrated in combination with other antiretroviral drugs [295]. Lopinavir (LPV, ABT-378, CID: 92727) is another peptidomimetic PI developed by Abbott Laboratories in 1998 [297]. LPV also has pharmacokinetic enhancer properties, as is the case for RTV, and is used as lopinavir/ritonavir fixed dose treatment for HIV/AIDS [295]. Darunavir [DRV, TMC114, (3*R*,3a*S*,6a*R*)-{3-[(4-aminobenzenesulfonyl)isobutyl-amino]-1-benzyl-2-hydroxypropyl}carbamic Acid Hexahy-drofuro[2,3-b]furan-3-yl Ester, CID: 213039, Fig. 7G] is a non peptidic PI developed by Tibotec BVBA in 2005 [298]. DVR is effective against mutant HIV-proteases that are resistant to PIs [292]. DRV is therapeutically used in combination with RTV or cobicistat (GS-9350, CID: 25151504, a cytochrome P450 3A inhibitor) [286,292,295].

8. Future perspectives and final remarks

As described in previous sections, the most usual strategies for new antiviral development includes high-throughput small-

molecule screening platforms and structure-based designs for targeting viral replication mechanisms. However, another interesting approach would be the use of therapeutic interfering particles (TIP) [299]. In this strategy, defective interfering particles (DIP) or antibody-recognizable capsid proteins could be employed in order to increase the susceptibility of the virus to be neutralized by the host immune system. Since genetic diversity and emergence of resistant viral variants is produced by the error-prone replication of the viral genome, this approach exploits the properties of the proteins that besides being functional (are able to form capsids), are not subjected to selective replication pressure. In this way, resistant and non-resistant genomes will be enveloped by chimeric capsids containing DIP and normal capsid proteins. For that strategy, direct administration of DIP proteins, or genetically engineered virus (which genomes encode DIP) can be employed for the treatment of viral infections. However, several safety concerns should be studied, such as the uncontrolled transmission of modified virus, the possible generation of unexpected and more virulent strains or the oncogenic transformation of cells.

One successful example of a non-antiviral drug modification is Didehydro-Cortistatin A (dCA) [300], an analog of the natural steroidal alkaloid Cortistatin A (CID: 11561907, CAS: 882976-95-6) produced by the marine sponge *Corticium simplex*. dCA is a potent suppressor of Tat-dependent HIV transcription, inhibiting HIV-1 and HIV-2 replication (Fig. 1.4a) at nanomolar levels. In addition to these properties, dCA inhibits viral release from latently infected $CD4^{+}$T cells isolated from highly active antiretroviral therapy (HAART) treated patients, suggesting that this compound can be considered as an alternative to the "shock and kill" strategy.

In a similar manner to the treatment of HIV, HCV or influenza virus, where the employment of drug cocktails is common, other antiviral treatments should have a holistic approach in order to reduce the appearance of resistant strains, allowing an eventual eradication of viral particles or infected cells in the organism. In addition to the combined use of antivirals, adjunctive therapies [301] are based on the use of antivirals combined with other non-antiviral drugs (e.g. hyaluronic acid [202]), that can be employed in order to reduce side effects, increase the bioavailability or tissue delivery (e.g. brain, cerebrospinal fluid, placenta ...) of active compounds, paying special attention to the implementation of personalized medicine. Similar results can be also obtained by the conjugation of antivirals with ligands that allow tissue targeting [286], such as lipids for liver accumulation, polyethylene glycol in order to increase stability, peptides and protein domains for increasing activity [301], among others.

The development of new antiviral compounds comprises several approaches, among which we can emphasize the study of new antiviral targets, unraveling virus and viral cycles, guide triggering of immune system response, modification of existing sources and their combinatory use, or the rediscovery of old drugs with new functions, among others. All those factors must be taken into account, since the main part of viral resistances is found after the treatment with only one drug. Indeed, several of the antivirals reported in this work, especially the ancient ones, have been studied from a result-oriented point of view, while their mechanisms of actions often remain unclear. Regardless of the elapsed time, or the discovery of new drugs, it is important to notice that we still have a large amount of antivirals that are yet to be employed, or that have been forgotten by scientific community.

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