



Review

The viral RNA capping machinery as a target for antiviral drugs

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ABSTRACT

Most viruses modify their genomic and mRNA 5'-ends with the addition of an RNA cap, allowing efficient mRNA translation, limiting degradation by cellular 5'–3' exonucleases, and avoiding its recognition as foreign RNA by the host cell. Viral RNA caps can be synthesized or acquired through the use of a capping machinery which exhibits a significant diversity in organization, structure and mechanism relative to that of their cellular host. Therefore, viral RNA capping has emerged as an interesting field for antiviral drug design. Here, we review the different pathways and mechanisms used to produce viral mRNA 5'-caps, and present current structures, mechanisms, and inhibitors known to act on viral RNA capping.

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1. Introduction

In the eukaryotic cell, RNA capping is a co-transcriptional event consisting of a chemical modification of the nascent mRNA 5'-end. Since the early 70s, viruses have played a pivotal role in the discovery and structural characterization of the RNA cap (Fig. 1), as well as in the mechanistic elucidation of the RNA capping pathway. Incidentally, virus families for which these discoveries were made (*Reoviridae* and *Poxviridae*) use an RNA capping pathway that turned out to be the same as that of their eukaryotic hosts. Soon after these discoveries, other virus families were found to deviate substantially from this 'conventional' pathway (see below).

In the conventional pathway, a cap structure is added to the nascent 5'-triphosphate mRNA in a series of reactions (Fig. 2). The 5'-triphosphate is first hydrolysed by an RNA 5'-triphosphatase (RTPase). A guanylyltransferase (GTase), also called "capping enzyme", adds the cap structure under the form of a guanosine 5'-monophosphate in a 5'-5' orientation. The cap is then methylated onto the N-7 position of its guanine by an RNA cap guanine N-7-methyltransferase (N-7 MTase). This generates the minimal cap-0 (m7GpppN...), found in metazoan and lower eukaryotes. In higher eukaryotes, further methylation by ribose 2'-O-methyltransferases (2'-O MTases) occurs at the 2'-position of the riboses of the original transcript to yield mainly cap-1 (m7GpppNmN...) but also cap-2 (m7GpppNmNmN...) structures.

The presence of the cap ensures stability of the transcript against a variety of cellular 5'-3' exonucleases and recognition of the mRNA by the ribosomal protein eIF4E for efficient translation. The capping was also shown to be involved in other cellular processes such as RNA splicing and export (Darnell, 1979; Filipowicz et al., 1976; Schibler and Perry, 1977).

Because of its coupling to RNA transcription, RNA capping is mainly a nuclear process, although some RNA re-capping events are suspected to occur in the cytoplasm (reviewed in Schoenberg and Maquat (2009)). Viruses generally replicate in the cytoplasm, causing there to be a time-window during which viral RNAs are synthesized but not yet capped. Virus and cell co-evolution has generated a number of cellular pathways and proteins involved in sensing the presence of viral RNAs. The absence of RNA cap as well as the presence of double stranded RNA are strong tokens

for a viral infection. These RNA species, alone or together, are detected as "non-self" RNA by cellular sensors triggering an innate cellular immunity response (Koyama et al., 2008; Takeuchi and Akira, 2007; Wilkins and Gale, 2010). Viruses have evolved numerous strategies to escape detection, including rapid and efficient viral RNA capping.

Few virus families and genera do not rely on RNA capping. *Picornaviridae* use a protein as an RNA synthesis primer and this protein replaces the RNA cap in its role for transcription promotion and RNA protection. Viruses from *Pestivirus* and *Hepacivirus* genera use unprotected 5'-triphosphate RNA ends and other strategies to defend their RNA from the cell immunity systems (Garaigorta and Chisari, 2009; Guidotti and Chisari, 2001; Malmgaard, 2004).

However, the vast majority of viruses use RNA capping. With the ongoing deciphering of viral RNA capping machineries, a true diversity of mechanisms, partners, and pathway organizations which invariably leads to the same RNA structure are progressively being uncovered. This diversity and its differences from the cellular RNA capping machineries are drawing a lot of attention for antiviral drug design.

2. Is RNA capping an appropriate target for antiviral research?

There are factors to bear in mind before considering RNA capping as an interesting drug design target:

Specificity: a most often put forward requirement is the uniqueness of the viral target, i.e., the non-existence of a similar cellular target that could also be hit by any antiviral drug and cause serious side-effects. Interestingly, even when viral enzymes remain close in structure and mechanism to their cellular counterpart, there remain structural and functional differences potentially useful to achieve differential inhibition, i.e., selectivity for the viral target. In most cases, viral enzymes are profoundly original in folding, organization, and mechanisms, providing a large chemical space for drug design and drug selectivity.

Potency: another important parameter is the expected outcome of viral target inhibition. The question of whether the *in vitro* inhibition effectively leads to a significant block of viral growth must be answered. One has to consider the two major mechanisms of action of the viral target. It can be an enzyme, and inhibition of its enzyme

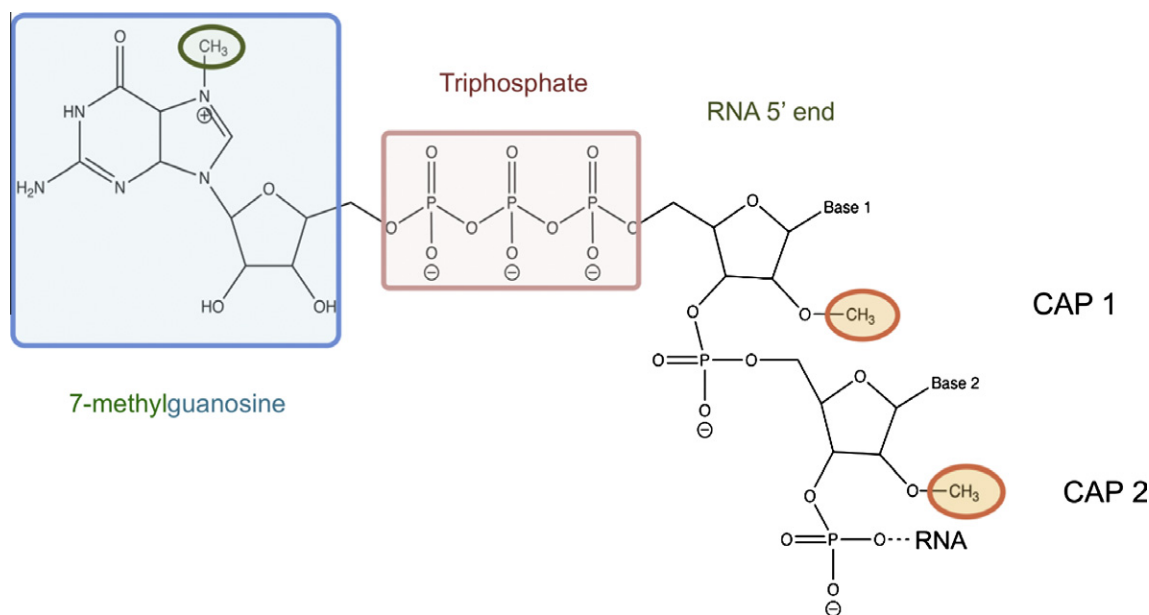


Fig. 1. The RNA cap structure: the cap consists of a 7-methylguanosine (blue box) linked to the 5' nucleoside of the messenger RNA chain through a 5'-5' triphosphate bridge (pink box). The methyl group of the guanosine at its N-7 position is surrounded in green, and the 2'-O methyl group of the first and second nucleotide residue forming the cap-1 and the cap2 structures, respectively, are surrounded in light orange.

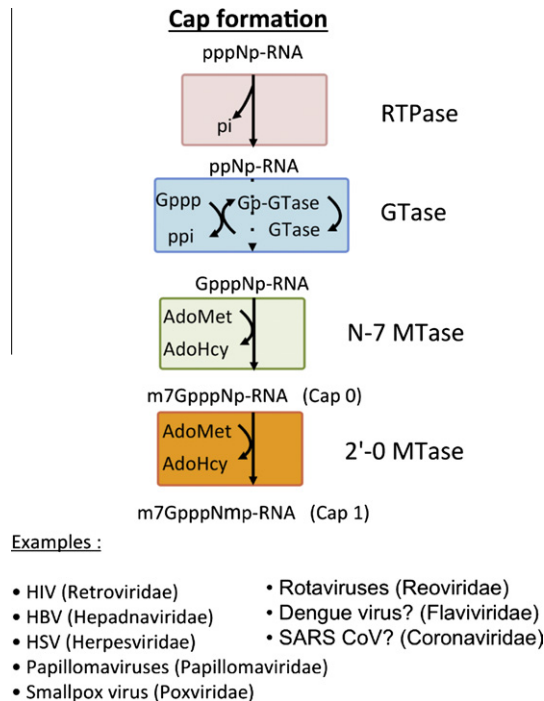


Fig. 2. Canonical RNA capping mechanisms. The cap-0 structure is formed on nascent RNA chains by the sequential action of three enzymes. (1) The RNA triphosphatase (RTPase, pink) hydrolyses the γ phosphate of the nascent RNA (pppN-RNA, where N denotes the first transcribed nucleotide) to yield a diphosphate RNA (ppN-RNA) and inorganic phosphate (P_i). (2) RNA guanylyltransferase (GTase, light blue) reacts with the α phosphate of GTP releasing pyrophosphate (PP_i) and forms a covalent enzyme–guanylate intermediate (Gp-GTase). The GTase then transfers the GMP molecule (Gp) to the 5' diphosphate RNA to create GpppN-RNA. (3) RNA (guanine-N-7)-methyltransferase (N-7 MTase, green), recruited by the GTase, transfers the methyl group from S-adenosyl-L-methionine (AdoMet) to the cap guanine to form the cap-0 structure (m^7 GpppN) and releases S-adenosyl-L-homocysteine (AdoHcy) as a by-product. The capping reaction is then completed by the methylation of the ribose-2'-O position of the first nucleotide by the AdoMet-dependent (nucleoside-2'-O)-methyltransferase (2'-O MTase, orange), generating cap-1 structure (m^7 GpppN_{m2'O}). Below the schematic are listed examples of viruses that acquire their cap structures using the cellular capping machinery (first four examples) or encode their own viral capping machinery that adopt the canonic pathway. Question marks indicate viruses likely to follow this conventional pathway.

activity may have exponential consequences. On the other hand, the virus may develop alternative pathways in order to resist antiviral molecules. For example, *Flavivirus* capping inhibitors had to be validated since dengue virus was also shown to perform cap-independent translation of its RNA genome (Edgil et al., 2006). Another possibility is that the target protein can be a binding partner devoid of catalytic activity and part of a protein–protein binding equilibrium. Its inhibition would shift the equilibrium according to the law of mass action. The protein dosage must be fine tuned to exert a powerful antiviral effect. In both cases, a significant effect on viral growth must be observed to validate the target as appropriate.

Amplifying mechanism: lastly, one has to consider the number of events that the protein or target is involved in during the virus life-cycle. For example, RNA-dependent RNA polymerases (RdRp) incorporate several thousand nucleotides to produce a single RNA genome. Inhibition of the viral RdRp at each nucleotide incorporation should exhibit a powerful antiviral effect. In contrast, RNA capping events can vary from a single capping event (e.g., the genome of ss(+)RNA viruses), to many RNA capping events (e.g., *Nidovirales*, *Mononegavirales*, etc.). Nevertheless, the connection between RNA capping and antiviral innate immunity pathways is expected to amplify any drug action on RNA capping. Immature or incomplete RNA caps may not only exhibit an altered expression profile, but may also be detected by a variety of innate immunity

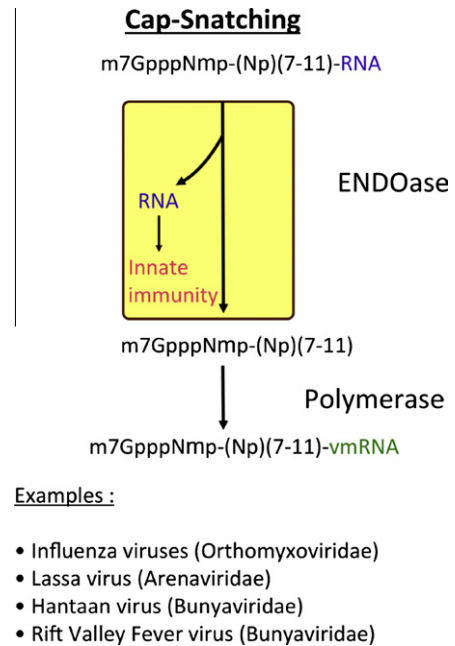


Fig. 3. Cap-snatching mechanisms. The endonuclease captures the cellular mRNA from the cell (blue) and snatches (cleaves and transfers) the cap structure followed by seven to eleven bases to the polymerase to start the viral mRNA (green). The residual cellular RNA serves as decoy for the innate immunity. The RNAs capped by viral enzymes are undistinguishable from cellular mRNA and can thus be translated into proteins by the host-cell ribosomal machinery. Below the schematic are listed examples of viruses that acquire their cap structures using the cap snatching mechanism.

sensors that add another direct negative effect on viral growth. In this context, it is noteworthy that the efficacy of RNA capping inhibition has been demonstrated. The inhibition of *Flavivirus* MTase, which catalyzes two methylations per synthesis of an entire RNA genome, has been reported to be able to completely suppress viral replication (Dong et al., 2008a).

3. Conventional and unconventional viral RNA capping mechanisms

Alongside the discovery of conventional cellular and viral RNA capping (Fig. 2), few viruses provided remarkable exceptions to the seemingly ubiquitous presence of capped mRNAs in Eukarya (as mentioned above for picorna-, hepaci- and pestiviruses). It was soon discovered that other viruses also used 'unconventional' RNA capping reactions deviating from the conventional RNA capping scheme (example in Fig. 3 and for complete review (Decroly et al., 2012)). Nevertheless, it is truly remarkable that several of these widely different RNA capping reactions and pathways converge to the same RNA cap structure (Fig. 4). This observation indicates that the evolutionary pressure to keep this structure protecting RNA must be significant. Viral RNA capping is thus an interesting target for drug design. In the following sections we summarize our current knowledge on viral enzymes involved in conventional and unconventional RNA capping (main reference structures in Fig. 5) and reported inhibitors (Fig. 6).

4. Conventional viral RNA cap-synthesizing enzymes

4.1. RNA triphosphatases

RTPases (Fig. 5, RTPase) are the enzymes responsible for the first step of cap formation, hydrolyzing the γ - β phosphodiester bond of

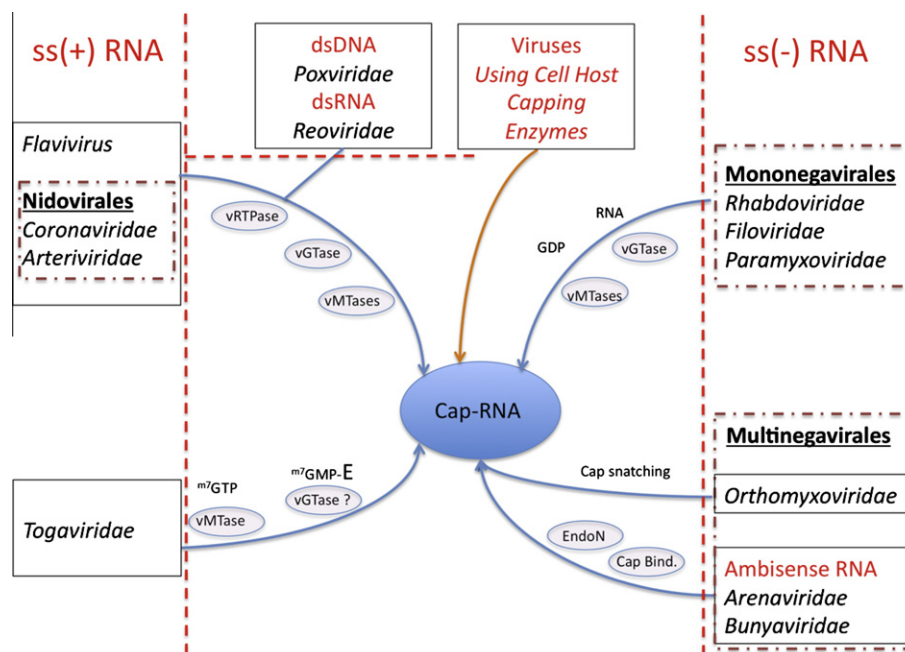


Fig. 4. Viral RNA capping pathways. Mammalian viruses, with the exception of ssRNA(+) pestiviruses and ssRNA(+) hepaciviruses, use strategies to chemically modify their mRNA 5'-ends through either covalent attachment of a protein (Viral Protein genome-linked, VPg, for ssRNA(+) -Picornaviridae, -Caliciviridae, and -Astroviridae), or covalent attachment of an RNA cap structure (all other viruses). When possible the viruses were divided by red dashed lines according to the nature of their viral genome (single stranded, double stranded, DNA, or RNA), and virus families and genera are written in italics. Three virus orders (ssRNA(+) *Nidovirales* and ssRNA(-) *Mononegavirales* and *Multinegavirales* (ssRNA(-) and ambisense RNA) are shown in bold and underlined. Enzymes and mechanisms are indicated along arrows leading to Cap-RNA. Small letters "v" ahead of the enzyme name indicate that the enzyme is of viral origin. The list of presented families is non exhaustive and used as an example.

5'-triphosphorylated mRNA. There is no enzyme conservation amongst different kingdoms of life, meaning that the activity can be found within a domain or a single protein presenting structural and mechanistic diversities (for detailed review Decroly et al. (2012)). These diversities make the RTPase an attractive target for the development of specific and selective inhibitors. RTPases can indeed be separated into two classes sorted by mechanistic criteria, i.e., either with a metal-independent and -dependent mechanism (Gu and Lima, 2005; Shuman, 2002). This separation also divides higher eucaryote RTPases (including human) from the vast majority of viral RTPases.

The metal-independent RTPase is so far a lone structural family, including human RTPase, higher eukaryotes (plants) and viruses like baculovirus. The substrate binding and catalytic site of these enzymes is located in a P-loop enriched with histidines and a cysteine that mediates a two-step reaction with a covalent phosphoenzyme intermediate (Changela et al., 2001; Denu and Dixon, 1998).

Alternatively, for viral metal-dependent RTPases a structural and mechanistic variety was found over the years. Metal-dependent RTPases can be organized into three superfamilies: the HIT-like fold (α - β complex) (Jayaram et al., 2002), the 'triphosphate tunnel metalloenzyme' (TTM) (Benarroch et al., 2008; Gu and Lima, 2005; Lima et al., 1999; Shuman, 2001), and the viral RNA helicase-like fold that carries one or more of the so-called Walker motifs (Benarroch et al., 2004b).

The HIT-like fold (Fig. 5, RTPase III) found in *Rotavirus* NSP2 protein has a magnesium dependent NTPase/RTPase hydrolysis activity capable of removing the γ -phosphate of either NTP or RNA (Jayaram et al., 2002). Both activities share the same catalytic site and mechanism. Also, the switch from one activity to the other is substrate dependent (NTP or pppRNA). NSP2 is a protein that self-assembles into a doughnut-shape octamer, which binds to single-stranded RNA after destabilizing RNA-RNA duplexes. Each NSP2 monomer has two subdomains separated by a deep electro-

positive cleft containing histidine residues involved in the binding and the hydrolysis of NTP as well as RNA (Vasquez-Del Carpio et al., 2006). The cleft is oriented on the surface of the doughnut. The N-terminal subdomain is mostly α -helical while the C-terminal domain adopts an α/β fold with a central twisted and anti-parallel β -sheet made of 5 β -strands, which is flanked by 5 α -helices. The protein is both a structural component of the RNA packaging and part of the capping machinery. Due to its unique structural features, it should be considered as a target of choice for the development of antiviral compounds.

Enzymes from the TTM superfamily hydrolyze NTPs to NDP + Pi in the presence of manganese or cobalt and are found in fungi, protozoa, and most of DNA viruses that encode a RTPase (*Orthopoxvirus*, *Chlorovirus*, *Baculoviridae* and *Mimivirus*). The RTPase of *Saccharomyces cerevisiae* Cet1 serves as a model (Fig. 5, RTPase I) and presents a structural tunnel composed of eight antiparallel β strands with a motif encompassing two glutamates (Lehman et al., 1999; Lima et al., 1999). The tunnel harbors several charged and hydrophilic side chains coordinating manganese and sulfate ions. The sulfate is thought to indicate the position of the γ -phosphate of the newly synthesized mRNA. In a docking/modeling-based study, a series of nucleoside analogues (6-chloropurine-ribose-5'-triphosphate, 6-methylthioguanosine-5'-triphosphate, or 8-iodo-guanosine-5'-triphosphate) was identified with high affinity for binding and resistant to hydrolysis (Despins et al., 2010; Is-sur et al., 2009a).

The last identified superfamily is the viral RNA helicase-like fold (Fig. 5, RTPase II), which carries NTPase-helicase activity. These RTPases are found in the RNA virus genera or families *Flavivirus* (NS3), *Coronaviridae*, *Orthoreovirus*, *Alphavirus* and *Potexvirus*. Structurally, they can belong to either SF1 or SF2 helicase superfamily. No viral helicase crystal structure belonging to the SF1 superfamily has been determined to date, yet the fold is thought to be divergent from the canonical SF1 (Cordin et al., 2006). On the other hand, the SF2 fold is formed by two RecA-like sub-do-

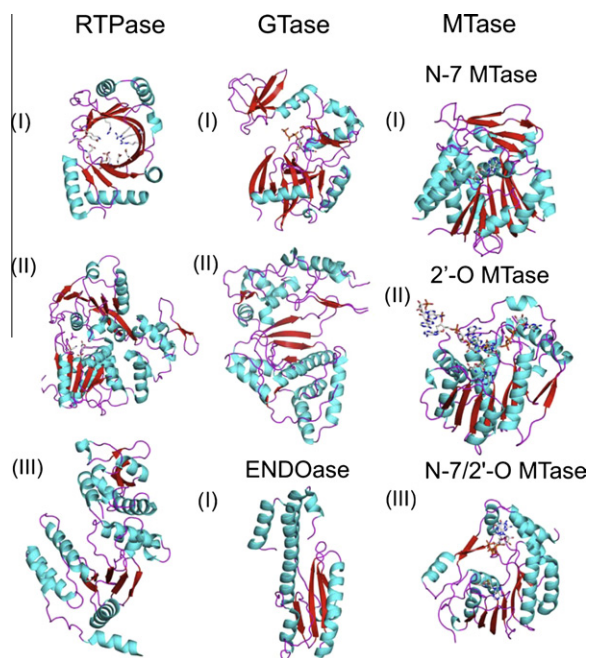


Fig. 5. Structural models of viral enzymes involved in RNA capping. **RTPase folds:** (I) the triphosphate tunnel metalloenzyme (TTM) fold is exemplified by the structure of the RTPase (PDB code 2QZE) from the genus *Mimivirus*, which consists of double-stranded DNA (dsDNA) viruses; (II) the helicase fold of the RTPase from the single-stranded positive-sense RNA (ss(+)RNA) dengue virus (PDB code 2BHR); (III) the histidine triad (HIT)-like fold of the RTPase from dsRNA rotaviruses (PDB code 1L9V). **GTase folds:** (I) Structure of the dsDNA virus *Paramesocystis bursaria* chlorella virus 1 GTase, showing the loading reaction: GTP onto the active site, with the GTase in an open conformation (PDB code 1CKO); (II) GTase domain of the Cypovirus (PDB code 3IZ3, residues 1–366). **MTases folds:** (I) N-7 MTase: the (guanine-N-7)-methyltransferase (N-7 MTase) domain of protein D1 from the dsDNA virus vaccinia virus (PDB accession code 2VDW) in complex with a molecule of S-adenosyl-L-homocysteine (AdoHcy). (II) 2'-O MTase: VP39, the 2'-O MTase of the dsDNA virus vaccinia virus, in complex with a capped RNA and AdoHcy (PDB code 1AV6). (III) N-7/2'-O methyltransferase: the N-7 MTase-(nucleoside-2'-O)-methyltransferase (2'-O MTase) domain of NS5 from dengue virus (ss(+)RNA) in complex with the cap analogue 7-methyl-G (m7G)-pppGm2'-O and AdoHcy (PDB code 2P41). **Endonuclease fold:** (I) endonuclease (N-terminal) domain of L protein of lymphocytic choriomeningitis virus (PDB code 3JSB). Structures are colored cyan for α -helices and red for β -strands. All figures were prepared using PyMOL.

main, separated by a cleft that can accommodate either a nucleotide or 5'-triphosphate RNA substrate. The two sub-domains carry the Walker motifs: the A motif forms the P-loop stabilizing the terminal phosphate moiety of the substrate, and the B motif is composed of acidic residues (D-E-X-D) coordinating the divalent ion needed for substrate hydrolysis.

All the catalytic sites described above, or clefts accommodating the RNA substrate or nucleotides could be used as a target for drug design. However, more efforts are needed to better understand the fine mechanistic details that would lead to rational drug design.

4.1.1. Inhibitors

Amongst the different families of viral RTPases, very few inhibition studies have identified potentially interesting compounds (Fig. 6). In that respect, the least neglected RTPase is cvRTP1, from the DNA *Paramesocystis bursaria* *Chlorella* virus 1. Not surprisingly, phosphate analogues (see Fig. 6, RTPase inhibitor I and II, also vanadate) are weak inhibitors, with no “drug-like” properties (Takagi et al., 2003). The family of *Flavivirus* NS3-like enzymes may have greater value, since in addition to the RTPase activity they harbour two other activities (helicase and NTPase). The RTPase active site is super-imposable to the NTPase active site, which provides energy to unwind dsRNA. Suppression of the RTPase/NTPase activity

abrogates helicase activity, therefore, it is likely that bi-functional inhibitors will be discovered in the near future (Lescar et al., 2008). One example for this approach has been a study of the inhibitory potential of purine analogues (one structure in Fig. 6, RTPase inhibitors III) that are expected to inhibit all three activities (Despins et al., 2010).

4.2. Guanylyltransferases

GTases (Fig. 5, GTase) are responsible for attaching a GMP molecule onto the pre-mRNA. Contrary to RTPases, fold and mechanism seem to be very well conserved amongst all kingdoms of life, making *de facto* the GTase a difficult target for specific inhibitor development. Yet there is a possibility that in the viral kingdom, GTases exhibit other mechanisms and new folds that would make GTases good candidates for drug development. This assumption comes from both the difficulty in identifying GTases in the viral world and the presence of GTase domains embedded within larger proteins such as protein L in Mononegavirales or the variant GTase domain found in cypovirus (Cheng et al., 2011) (Fig. 5, GTase II). Known GTase proteins so far adopt a modular organization containing an N-terminal nucleotide transferase domain with an ATP-grasp fold and a C-terminal domain with an oligonucleotide/oligosaccharide binding fold (OB-fold) that is positioned as a lid over the base subdomain of the N-terminal domain (Fig. 5, GTase I). The GTP-binding site is located between the base and the hinge, and is highly conserved in GTases from dsDNA viruses to humans. The catalytic site can be defined by the presence of six conserved motifs, for which Motif I contains a lysine that covalently links GMP by hydrolysis of ppi from GTP, before its transfer onto RNA. Other conserved motifs form the binding pocket for the nucleotide. GTases from the dsRNA viruses of the *Orthoreovirus* genus form part of a multidomain protein (or assembly line) carrying all RNA capping functions. The GTase domain probably exists transiently and no fold can be clearly defined, whereas the methyltransferase domains are identifiable. Nevertheless, the catalytic residue is also a conserved lysine (Sutton et al., 2007).

4.2.1. Inhibitors

Very few compounds have been described that could provide a useful start for drug design. In the case of DNA viruses, the enzyme shares structural and functional similarities to the ligase protein superfamily, raising potential difficulties in achieving selectivity inside the host cell. The pyrophosphate analogue foscarnet (a phosphonic acid derivative (Fig. 6, GTase inhibitor I)) has been shown to inhibit the reaction, not surprisingly since the reaction produces pyrophosphate (Soulière et al., 2008). In the case of several viral families the GTase is still unknown (coronaviruses, flaviviruses). In the flaviviruses, the GTase activity might be expressed by the N-terminal domain of protein NS5 carrying also other related RNA capping activities (MTases, Issur et al., 2009b). Therefore, structural and mechanistic studies may provide efficient drug leads targeting several activities at once as it is the case for Ribavirin, a guanine nucleotide analogue, targeting GTase and MTase (Benarroch et al., 2004a; Bougie and Bisailon, 2004).

4.3. Methyltransferases

The last step of the cap formation consists in the methylation of the cap by RNA cap methyltransferases (Fig. 5, MTase). The N-7 position of guanine is methylated by the N-7 MTase and the first nucleotide of the RNA transcript is further methylated at the ribose 2'-OH position by 2'-O MTase (see Fig. 1). S-adenosyl-L-methionine (SAM) is the methyl donor for both the N-7 and 2'-O methylations, generating S-adenosyl-L-homocysteine (SAH) as a by-product. The two methylations are either done by specific proteins (or domains)

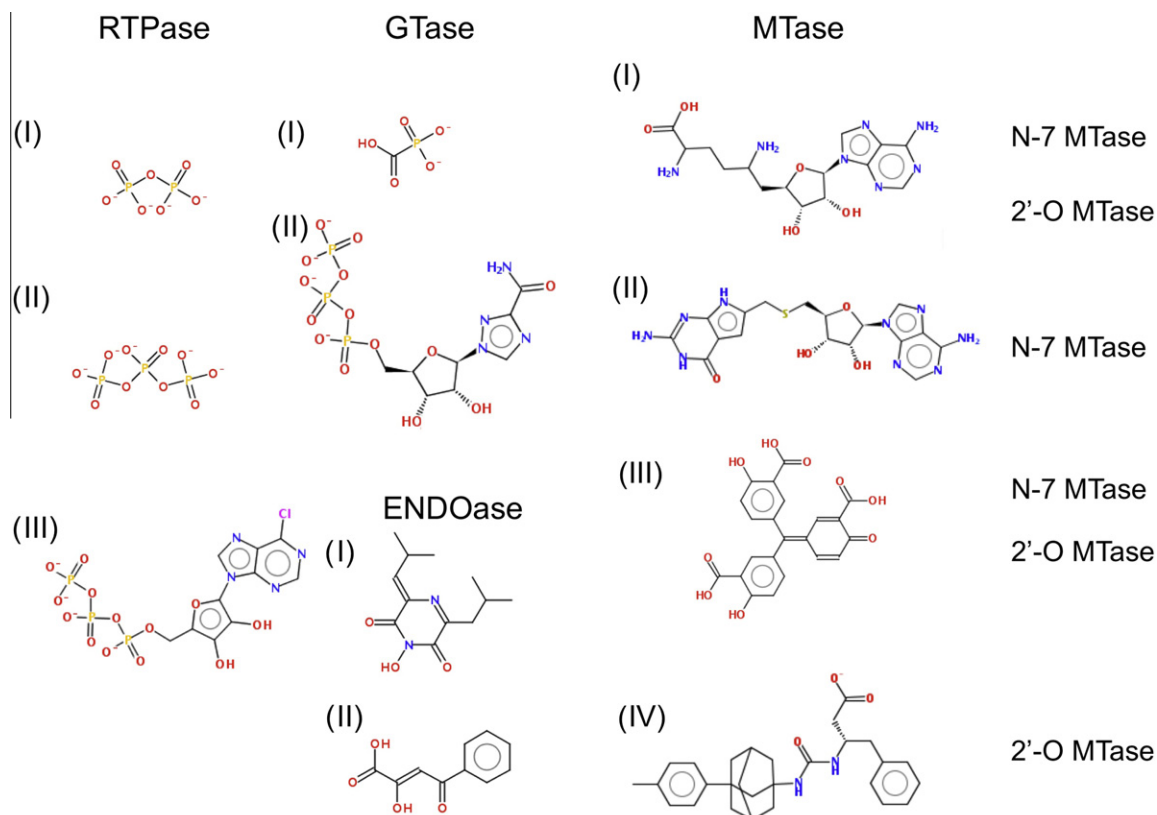


Fig. 6. Capping enzymes inhibitors. Inhibitors structure examples. *RTPase inhibitors:* (I) PPi (II) PPPi (III) 6-chloropurine-ribose-5'-triphosphate. *GTase inhibitors:* (I) foscarnet (II) sinefungin. *MTase inhibitors:* (I) sinefungin (II) dAPPMA 2'-O MTase inhibitors: (III) aurintricarboxylic acid (IV) inhibitor 7. *Endonuclease inhibitors:* (I) DPBA (II) Flutimide.

(Poxviridae, Coronaviridae, Reoviridae) or by one single protein (or domain) (Flavivirus).

The canonical SAM-dependent MTase fold presents alternating β strands and α helices, that form a seven-stranded β sheet with at least three parallel α helices on each side (α/β fold). The structure is reminiscent of the Rossmann fold also found in the dinucleotide-binding domains of dinucleotide-binding proteins. The SAM-binding region is located at the N-terminal part of the β sheet and is formed in part by residues from loops following the central strands of the sheet. The substrate-binding region that is responsible for selectively binding nucleic acids is located in the C-terminal part of the β sheet. Depending on nature of the methylation (N-7 or 2'-O or both) additional structural features can be found to help accommodate or stabilize the substrate.

The N-7 MTase of vaccinia virus (VV) serves as structural and reference model of N-7 MTases. The activity is carried by the complex of the C-terminal domain of protein D1 and protein D12. Both the domain of D1 and D12 present the characteristic core α/β fold of the MTase family (Fig. 5, N-7 MTase I), but the D12 protein lacks a proper SAM binding site and does not show any activity on its own. In contrast, it stimulates the MTase activity of D1 by 30- to 50-fold. D12 does not affect the extent of substrate binding to the catalytic subunit. Rather, it has an allosteric role increasing the affinity for ligands as well as increasing the overall stability of the complex. Recently, similar finding have shown that the SARS 2'-O MTase activity harbored in protein nsp16 is stimulated by a small regulatory protein nsp10 (Bouvet et al., 2010; Decroly et al., 2011).

VP39 protein of VV serves as reference for 2'-O MTase structures. It is a single-domain protein with a typical α/β fold (Fig. 5 2'-O MTase II). This structure has all the features of the canonical core domain. The catalytic residues of 2'-O MTases are defined by

a conserved tetrad motif K-D-K-E located at the interface of SAM-binding cavity and the substrate-binding cleft (Fig. 5, 2'-O MTase II). VP39 exhibits a hydrophobic pocket, at the end of the RNA binding cleft, which presents aromatic side chain residues involved in stacking in the capped RNA.

Single domain MTases are exemplified by the *Flavivirus* MTase domain of protein NS5. The NS5 protein of flaviviruses is divided into two domains, an MTase residing in the N-terminal (one-third of NS5) and an RNA-dependent RNA polymerase domain in the C-terminal (two-thirds of NS5). The NS5 MTase domain was first unambiguously characterized as a 2'-O MTase (Egloff et al., 2002) and later it was shown that the same SAM-binding site is used for N-7 MTase activity (Ray et al., 2006). The core structure of the domain consists of the 7-stranded β -sheet surrounded by four α -helices (Fig. 5, N-7/2'-O MTase III) and its catalytic site is identified by the universal tetrad K-D-K-E (Fig. 5, N-7/2'-O MTase III). The core of the structure is not supposed to rearrange, yet optimal N-7 and 2'-O methylations of the flavivirus cap require distinct biochemical conditions, suggesting that the two methylations occur through different mechanisms. Mutagenesis of the tetrad has shown that only the D of the K-D-K-E catalytic tetrad is essential for the N-7 methylation, and structural studies are still investigating the determination of the complete catalytic site for the N-7 MTase. Ray and colleagues (Ray et al., 2006) have shown that the two cap methylation events are sequential and that the reaction is controlled by a steric constraint for the substrates. Indeed the N-7 methylation requires wild-type nucleotides at the 2nd (G) and 3rd (U) positions, with a 5'-stem-loop structure, whilst the subsequent 2'-O methylation requires wild-type nucleotides at the 1st (A) and 2nd (G) positions and a minimum of 20 nucleotides of viral RNA.

4.3.1. Inhibitors

The exploration of MTases constitutes the most actively growing field amongst viral RNA capping targets. The last 10 years have seen viral RNA cap MTases coming increasingly into focus as targets for the discovery and development of antivirals (Dong et al., 2008b; Issur et al., 2011; Liu et al., 2010). The inhibition of N-7 MTase activity is expected to exert a deleterious effect on viral replication because it blocks viral RNA translation. The essentiality of the N-7-methylation for viral replication has clearly been demonstrated for several viral families (Almazan et al., 2006; Dong et al., 2008a; Ray et al., 2006; Zhou et al., 2007). In contrast, mutational analysis indicates that 2'-O MTase inhibition has weak effects on virus replication in cell culture (Ray et al., 2006; Zhou et al., 2007). Nevertheless, 2'-O MTases are now also considered as potential targets due to the demonstration that RNA, lacking 2'-O-methylation on their cap structure, induces antiviral response through the MDA5 sensor (Züst et al., 2011). Viruses mutated in their 2'-O MTase gene accordingly show strongly reduced replication capacity in animal models (Daffis et al., 2010). This has led to a re-evaluation of the importance of 2'-O-methylation (reviewed in (Decroly et al., 2012)).

The discovery of inhibitors of viral MTases started and the elucidation of RNA capping started in the dsDNA *Poxviridae* field. A SAM analogue, sinefungin (Fig. 6, MTase inhibitor I), was reported as the first potent inhibitor of the VV N-7 MTase activity (Pugh et al., 1978) with a potent antiviral effect shown in a plaque formation assay. Another early study described inhibition of N-7 MTase and 2'-O MTase activities of VV by 2',5'-linked oligo(adenylic acid) triphosphates (Sharma and Goswami, 1981). Interestingly, these molecules are “natural” metabolites produced by the host cell as part of the innate immune response against viral infection (reviewed in Decroly et al. (2012)). Later, multi substrate adducts with inhibitory activity against VV N-7 MTase were reported (Fig. 6, MTase inhibitor II) (Benghiat et al., 1986). SAH analogues were shown to inhibit VV and herpes simplex virus (HSV) and their respective MTases were proposed as potential molecular targets (Balzarini et al., 1992).

Concerning RNA virus MTases being part of the conventional capping machinery, *Flavivirus* and *Coronavirus* MTases have been particularly explored. The dengue virus 2'-O MTase activity was shown to be inhibited by the GTP-analogue ribavirin triphosphate (Benarroch et al., 2004a), sinefungin, and SAH (Selisko et al., 2010). Based on known complex structures of the dengue virus NS5 MTase domain (Benarroch et al., 2004a; Egloff et al., 2007, 2002) which define the RNA cap binding sites during 2'-O-methylation and N-7-methylation (the latter being the actual active site) as well as the SAM-binding site, several groups have employed virtual screening and docking procedures to identify potential ligands and thus inhibitors (Lim et al., 2011; Luzhkov et al., 2007; Milani et al., 2009; Podvinec et al., 2010). Some molecules were found to present inhibitory activity in the micromolar range in *in vitro* enzymatic assays (Luzhkov et al., 2007; Milani et al., 2009; Podvinec et al., 2010), see example in Fig. 6, MTase inhibitor IV). A new conserved hydrophobic binding pocket next to the SAM-binding site was identified on *Flavivirus* MTases, which is critical for viral replication and cap methylations (Dong et al., 2010). This invites to the design of SAM analogues that also interact with this adjacent hydrophobic site and should be thus more potent and specific in comparison to SAM analogues as sinefungin. High-throughput screens using MTase activity assays and GTP-binding assays were set up and used to identify inhibitor molecules (Geiss et al., 2011; Lim et al., 2008). Concerning *Coronavirus* MTases the search for antivirals has been less active. Some SAM analogues have been tested and found to inhibit the N-7 MTase activity of nsp14 and the 2'-O MTase activity of nsp16 in the low micromolar range (Bouvet et al., 2010). One of the identified molecules, aurintricarboxylic

acid (ATA, (Fig. 6, MTase inhibitor III)), also inhibits SARS-CoV replication in infected cells (He et al., 2004). An indirect approach to inhibit viral MTases is being considered for *Coronavirus* 2'-O MTase nsp16, the activity of which depends on its interaction with the nsp10 protein (Bouvet et al., 2010; Decroly et al., 2011; Lugari et al., 2010). Molecules that disrupt the interaction are expected to inhibit the MTase activity of nsp16 and thus viral replication. This approach can also be considered for the N-7 MTase activity of protein D1 of VV, which depends on the activation by the stimulatory protein D12 (De la Peña et al., 2007).

4.4. Cap assembly lines

Cap-assembly lines are proteins found in several dsRNA viruses (*Reoviridae*). They are multi-domain and function proteins that are packaged with the genome in the viral particle and are able to perform the four reactions needed to synthesize a cap-1 structure. Domains of the cap assembly line must be tightly regulated and coordinated to accomplish the sequential steps of the cap synthesis pathway as they can exhibit activity independent from or in synergy with the capping process. The individual domains corresponding to GTase and MTase have been characterized and have similar folds to those previously described, although it is unclear how and when RTPase activity occurs. A complete pathway has been proposed in which guanylyl transfer is followed by N-7-methylation and 2'-O-methylation of the mRNA.

Each domain/function could be targeted individually. The entire protein is also to be considered, as there is room for specific development to sterically impair the necessary dynamic movements of the respective domains. So far, though, no specific inhibitors of cap assembly lines or either of their associated enzyme activities have been described.

5. Unconventional cap synthesis pathways

The first indication for the existence of deviations from the conventional viral RNA capping pathway came in the early 1970s, around the time of the discovery of the RNA cap structure. Since then, it has been demonstrated that vesicular stomatitis virus (VSV, *Mononegavirales*) and alphaviruses (*Togaviridae*) can synthesize a viral RNA cap that is identical to a cellular RNA cap, albeit through two completely different mechanisms. *Togaviridae* do not proceed further than synthesizing a cap-0 structure, which remains an interesting enigma since many members of this family productively infect both insects and mammals.

5.1. The *Mononegavirales* RNA capping pathway

The *Mononegavirales* order, also referred to as negative-strand (–) non-segmented (NNS) RNA viruses comprises major human pathogens such as rabies virus (*Rhabdoviridae*), measles virus (*Paramyxovirinae*), bornavirus (*Bornaviridae*) and Ebola/Marburg viruses (*Filoviridae*). RNA capping is achieved by the multifunctional L protein, which carries both RNA-dependent RNA polymerase (RdRp) and RNA cap synthesis activities. Shortly after the discovery of the RNA cap and conventional capping pathway, it was observed that the VSV L protein (Abraham et al., 1975a, b) transferred GDP rather than GMP onto the nascent transcript. Subsequently, L proteins from spring viremia of carp virus (Gupta and Roy, 1980), human respiratory syncytial virus (Barik, 1993), and chandipura virus (Ogino and Banerjee, 2010) were shown to perform a similar reaction and presumably follow the same RNA capping pathway.

This discovery adds a novel enzyme to the process as the pathway involves a unique L-encoded GDP polyribonucleotidyltransfer-

ase activity (PRNTase), which forms a covalent enzyme-p-RNA intermediate involving a phosphoamidate bond. A conserved catalytic histidine is present in a “HR” motif instead of lysine used by conventional GTases (Ogino and Banerjee, 2010). GDP generated from GTP (Ogino and Banerjee, 2007) by a yet-unknown NTPase is then transferred to the 5'-monophosphorylated viral mRNA 5'-end covalently attached to the PRNTase. Interestingly, the methylation sequence also is different from the conventional pathway sequence. The RNA cap is first methylated at the ribose 2'-O position of the first nucleotide followed by the guanine N-7 position, generating an RNA cap-1 structure. No crystal structure is available yet to guide drug design against these unique enzymes. The PRNTase should be considered as a prime target, perhaps more amenable to antiviral design than the more common MTase fold.

5.1.1. Inhibitors

Most effort for antiviral design has been made for RSV, for which a sizeable market exists. Interestingly, two compound families having submicromolar activities (IC_{50} = 21–200 nM) have been described (Liuzzi et al., 2005; Sudo et al., 2005) that target the L protein. The most potent compounds of respective series have been used to elicit resistance and the resistance mutation map to an L region or domain consistent with the presence of the PRNTase. Although the PRNTase was discovered after the publication of these compounds, mechanistic studies clearly indicated that the guanylation step was the actual target of the compounds. For RSV, only two products are approved for antiviral therapy or prevention: a monoclonal antibody (Palivizumab, Synagis) and ribavirin (Empey et al., 2010; Vigant and Lee, 2011). The latter served as a control compound with moderate antiviral effect in the discovery of PRNTase inhibitors, but the precise mechanism of action of ribavirin (i.e., capping, error catastrophe, IMP dehydrogenase, or else (Leyssen et al., 2006; Severson et al., 2003; Zhou et al., 2003)) against *Mononegavirales* remains to be investigated.

5.2. The ssRNA(+) *Togaviridae* (alpha-like) RNA capping pathway

The *Togaviridae* (ss(+)RNA viruses) as exemplified by some alphaviruses (Semliki Forest virus, Sindbis virus and chikungunya virus) synthesize their cap-0 structure through another non-conventional mechanism (Fig. 4). The enzymes presumably involved in the capping pathway are poorly characterized and their crystal structures are not yet available. Nevertheless, it is likely that capping begins with N-7-guanine methylation of a GTP molecule by the nsP1 N-7 MTase. This methylation is seemingly followed by the formation of a covalent m⁷GMP-enzyme complex involving a conserved histidine catalytic residue (Ahola et al., 1997). The N-7-methylation of GTP seems to precede the formation of the enzyme-GMP complex, since the guanylate intermediate is not observed in the absence of AdoMet (Ahola and Kääriäinen, 1995; Ahola et al., 1997). Whereas the m⁷GMP transfer onto the 5' end of viral RNA was not yet formally demonstrated, it is expected that the 5' end of viral RNA bears a diphosphate. The hydrolysis of the β - γ phosphate bond at the 5' end is mediated by the nsP2 RTPase domain (Vasiljeva et al., 2000). Brome mosaic virus replicase protein 1A (Ahola and Ahlquist, 1999), bamboo mosaic virus ORF1 protein (Li et al., 2001), tobacco mosaic virus P126 (Merits et al., 1999) and hepatitis E virus p110 (Magden et al., 2001) were reported to share properties with alphavirus N-7 MTases and GTases. Interestingly, Sindbis virus and Semliki Forest virus were reported more than 30 year ago to contain additional methyl groups attached to the exocyclic N2 of the cap structure (HsuChen and Dubin, 1976; van Duijn et al., 1986). The role of this methylation in virus replication is still unknown, but it is reminiscent to 2,2,7-trimethylguanosine (TMG) cap that is found on non-coding eukar-

yal RNAs such as small nuclear (sn), small nucleolar (sno) RNAs, and telomerase RNA (Busch et al., 1982; Seto et al., 1999).

5.2.1. Inhibitors

Ribavirin shows broad spectrum *in vitro* inhibitory activity against RNA viruses, through presumably different modes of action (Leyssen et al., 2006; Severson et al., 2003; Zhou et al., 2003). In the case of alphaviruses, resistance mutants to ribavirin were mapped into the GTase domain of the nsP1 protein (Scheidel et al., 1987; Scheidel and Stollar, 1991) suggesting ribavirin interferes with the GTase activity. Nevertheless, the ribavirin action mode remains controversial. Ribavirin also reduces the intracellular concentration of GTP (Leyssen et al., 2005) and/or stimulates the expression of interferon-stimulated genes such as that of protein MDA5 (Thomas et al., 2011), which is known to sense viral RNA devoid of 2'-O-methylation (Decroly et al., 2012; Züst et al., 2011).

In addition some efforts have focused on the identification of specific inhibitors targeting specifically the nsP1 capping activity. Several GTP analogues have been reported to inhibit Semliki forest virus MTase and GTase activities with K_i values below 100 μ M (Lampio et al., 1999).

6. Virus-mediated RNA cap ‘snatching’

6.1. Enzymes from the cap-snatching pathway

The cap snatching mechanism is a common and alternative viral strategy for cap formation. Instead of making its own RNA cap, the virus has a machinery to remove and transfer (snatch) the cap from host mRNA onto its own pre-mRNA (Fig. 3). Three major human pathogen families, *Arenaviridae*, *Bunyaviridae* and *Orthomyxoviridae* (Fig. 4), all of them ss(–)RNA viruses, have developed this strategy (Bouloy et al., 1978; Caton and Robertson, 1980; Plotch et al., 1979). The *Orthomyxoviridae* serves as a paradigm for both mechanistic and structural studies. In the case of the influenza virus, the replication complex is made of three proteins forming the polymerase complex (PA, PB1, PB2). Host cell mRNAs present in the cytoplasm are targeted and recruited by PB2 that presents the cap from the mRNA to the endonuclease domain (PA) to snip the cap off. Then the short capped RNA is used as primer to polymerize the viral RNA. The first structural endonuclease domain potentially involved in cap snatching was recently identified in both *Arenaviridae*, and *Bunyaviridae* L protein (Morin et al., 2010; Reguera et al., 2010) (ENDOase, Fig. 5). The endonuclease fold features four mixed β -strands forming a twisted sheet surrounded by seven α -helices (Fig. 5). The β sheet forms the bottom of a negatively charged cavity creating a binding site for divalent cations. Above it, a C-terminal helix with a positive patch creates another pocket to accommodate the RNA where the typical conserved PD...(D/E)XK nuclease motif defines the catalytic site.

A fourth example of cap snatching has recently been described in *Totiviridae* for a fungal virus (Fujimura and Esteban, 2011). However, in this case the Totivirus L-A transfers only m⁷GMP snatched from cellular mRNA. Hence, this mechanism lies somewhere between alphaviruses, which transfer m⁷GMP acquired from GTP, and “conventional” cap-snatching viruses described above, which transfer longer capped RNA oligonucleotides. As an additional similarity to alphaviruses, the Totivirus L-A cap-snatching enzyme makes a covalent m⁷GMP-enzyme intermediate through a histidine residue (Fujimura and Esteban, 2011; Ahola et al., 1997).

6.1.1. Inhibitors

Ribavirin again is an effective inhibitor of *Arenaviridae* (Olschlager et al., 2011) and *Bunyaviridae* (Livonesi et al., 2006; Severson

et al., 2003), but it has pleiotropic effects as discussed above and thus there is no direct evidence that the RNA cap snatching process is an actual target.

The first discovery of inhibitors of the cap snatching pathway was made in 1994, when Tomassini et al. reported that 2,4-dioxobutanoic acid compounds were able to inhibit Influenza A and B viruses in infected cell cultures (Tomassini et al., 1994). Remarkably, the compounds exhibited IC_{50} values in the micromolar range, comparable to IC_{50} values observed when purified polymerase cores were used in an *in vitro* endonuclease assay. The following chemical diversification of the parent compound could achieve a high specificity for influenza. Indeed, the L-735,882 compound reached an IC_{50} value of 1.1 μ M for influenza in an *in vitro* endonuclease assay and an IC_{50} value of 2 μ M in a viral titer assay using influenza virus infected cells. The La Crosse virus, a *Bunyaviridae* for which RNA capping and RNA polymerase priming rely also on cap snatching, remained unaffected both at the enzyme and virus level. Interestingly, the La Crosse virus replicates in the cytoplasm exclusively, unlike the influenza virus. Therefore, RNA caps are likely snatched in different cellular compartments or environments by these two viruses and this should be considered at early steps of drug design for large spectrum inhibitors.

In 2009 and 2010, a structural basis for the inhibition mechanisms was provided by the crystal structure of the La Crosse virus endonuclease domains in complex with DPBA (Reguera et al., 2010) (Fig. 6, ENDOase inhibitor I)). The structural homology between the three available crystal structures indicates that a common pharmacophore including Mn^{2+} chelating functional groups might be active against all three viral families. Based on these crystal structures, several compound families have been described and their antiviral activities characterized using enzyme-based assays, viral growth assays and protein structure data. The first compounds benefiting from structure-based activity analysis are catechins isolated from green tea. Although they seem to target both neuraminidase (Song et al., 2005) and endonuclease, one active pharmacophore was identified as 3,4-dihydroxyphenyl. The same group extended the family of interesting compounds with thalidomide derivatives, active through their 3,4-dihydroxyphenethyl moieties, and later with marchantin-like phytochemicals produced in very high yield in liverworts (Iwai et al., 2011). Together with enzyme and virus-infected cell assays, docking the latter compounds into the PA endonuclease active site gave very interesting perspectives for effective drugs targeting influenza RNA capping.

7. Conclusions

The viral RNA capping machinery is remarkably diverse in organization, structure, and mechanisms used by its enzymes. All RNA viruses capping their own viral RNAs have evolved enzymes profoundly different from those of their host cell. As the power of antiviral compound screening and design increases, there are no doubts that viral RNA capping enzymes will be the target of novel highly efficient and selective drugs. Despite few exceptions, the identification and atomic resolution structure of target enzymes from major families has made significant progress in the last ten years. The recent connection of viral RNA capping to the host cell innate immunity mechanisms is more than ever a promising antiviral research field.

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