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Biochemical principles and inhibitors to interfere with viral capping pathways
Etienne Decroly and Bruno Canard

Messenger RNAs are decorated by a cap structure, which is essential for their translation into proteins. Many viruses have developed strategies in order to cap their mRNAs. The cap is either synthesized by a subset of viral or cellular enzymes, or stolen from capped cellular mRNAs by viral endonucleases (‘cap-snatching’). Reverse genetic studies provide evidence that inhibition of viral enzymes belonging to the capping pathway leads to inhibition of virus replication. The replication defect results from reduced protein synthesis as well as from detection of incompletely capped RNAs by cellular innate immunity sensors. Thus, it is now admitted that capping enzymes are validated antiviral targets, as their inhibition will support an antiviral response in addition to the attenuation of viral mRNA translation. In this review, we describe the different viral enzymes involved in mRNA capping together with relevant inhibitors, and their biochemical features useful in inhibitor discovery.

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Introduction
The 5’ end of nascent eukaryotic messenger RNA (mRNA) is co-transcriptionally modified by the addition of a cap structure. The cap-0 structure consists of a guanosine linked by a 5’-5’ triphosphate bridge to the RNA 5’ end (Figure 1a). This cap structure is methylated at the nitrogen in position 7 of G (cap-0 structure or m7GpppN). In metazoan, cap-0 is often converted into cap-1 structure by 2’-O-methylation of the first N1 ribose (cap-1 structure or m7GpppN2,2’N) of the mRNA. This structure plays several key biological functions (reviewed in Ref. [1**]). The cap (i) increases mRNA stability by protecting mRNA from 5’ exoribonucleases; (ii) participates to pre-mRNA splicing and export to the cytoplasm; (iii) ensures the recruitment of mRNA to the ribosomes by recognizing eukaryotic translation Initiation Factor (eIF4E); and (iv) initiates the translation of mRNA into proteins. In addition, it was demonstrated that the cap structure is a marker of ‘self’, preventing detection by mechanisms of cellular innate immunity [2]. It was first reported that host cell sensors, such as Toll Like Receptor (TLR) and Retinoic acid-Inducible Gene (RIG)-like receptors, could detect uncapped RNAs with 5’-triphosphate ends. More recently, it was also shown that RIG-I and Melanoma Differentiation-Associated protein 5 (MDA5) recognize mis-capped RNA lacking 2’-O-methylation of the first transcribed nucleotide [3,4] initiating signaling cascades leading to the expression and release of cytokines and type I interferon. In turn, interferon induces an antiviral state in neighboring cells. Among the Interferon-Stimulated Genes (ISG), Interferon-Induced protein with Tetradricopeptide repeats 1 (IFIT 1) can recognize mis-capped RNAs and inhibit their translation [5].

Within the host cell, eukaryotic mRNA is generally capped through a ‘canonical’ RNA capping pathway. It generally requires four sequential reactions, elucidated four decades ago, catalyzed by an RNA 5’ triphosphatase (RTPase), a guanylyltransferase (GTase), a guanine N7 methyltransferase (N7-MTase) and a 2’-O-MTase, respectively (Figure 1b).

In contrast, many viruses have evolved their own mRNA capping machinery in order to expedite efficient viral protein production and escape from innate immunity detection. Remarkably, pathways of viral mRNA capping are highly diverse but almost converge to the RNA cap structure common to viral and cellular mRNAs (Figure 1a) [6*]. When viruses express their own set of capping enzymes, four types of RNA capping pathways have been evidenced so far [1**,6*].

In the first one, viruses use a capping pathway similar to that observed in eukaryotic cells (Figure 1b). The phosphate at the 5’ end of the nascent viral is hydrolyzed by an RTPase activity held by an RTPase or a helicase domain. Concomitantly, a GTP is recruited by a GTase, often forming a covalent adduct Lys-GMP before the transfer of GMP onto the RNA 5’ diphosphate end [7–11]. This occurs for DNA viruses (e.g., poxviruses, mimivirus,
baculoviruses) as well as supposedly for several positive strand RNA viruses (e.g., flavivirus, coronavirus). After capping, the cap is methylated on its N7 and 2’O position by either one bi-functional N7/2’O-MTases (e.g., flaviviruses [12,13]), or two separate enzymes (e.g., coronaviruses [14]).

The non-segmented negative strand (NNS) viruses use a distinct RNA capping pathway (Figure 2a). The most studied NNS, VSV, codes for a large (L) protein performing both replication/transcription and capping of viral RNA [15]. The cap synthesis is ensured by a polyribonucleotidyltransferase (PRNTase), which forms a covalent link between a conserved histidine and the nascent viral mRNA. In the presence of GDP, the cap structure is formed and the MTase domain in C terminus of the L protein methylates the cap structure at the ribose 2’O position of the first transcribed nucleotide, followed by the cap-guanine at its N7 position.

*Toxoviridae* also synthesize a cap structure using a non-conventional mechanism (Figure 2b). This virus family (and also bamboo mosaic virus, a plant pathogen from the related potexvirus genus) codes for an enzyme (alphavirus nsp1) that methylates the N7 of GTP and forms a covalent His-N7/GMP complex [16,17,18]. The
(a) Negative Non-Segmented (NNS) virus RNA capping pathway, in which the nascent viral mRNA is sequentially processed by four enzymatic activities, represented as separate enzymes on the right-side of the reaction. These enzyme activities are generally present in L, a single large polypeptide chain encompassing the viral RNA-dependent RNA polymerase. The NTPase generates a diffusible GDP molecule, and the spatial arrangement and cross-talk of PRNTase and NTPase is still unclear. (b) Togaviridae (alphavirus-like) RNA virus capping pathway. The N7-GTP MTase generates a diffusible m7GTP molecule, and here also, the spatial arrangement and cross-talk of the N7-GTP MTase and GTase is still unclear. (c) RNA cap-snatching pathway. Viral RNA-dependnet RNA polymerases (RdRp) have (or may have) an RNA cap-binding site in close proximity to an endonuclease (endoN) and distinct from the polymerase active site. The size (n) of the snatched capped primer varies within viral families (see text for details). Abbreviations as in Figure 1, plus the following: PRNTase: GDP Polynucleotidyl Transferase; NTPase: nucleoside 5'-triphosphate phosphatase; EndoN: endonuclease.

methylated GMP is then transferred onto the nascent viral RNA yielding a cap-0 structure. These viruses do not methylate the 2'O position of the first transcribed nucleotide, raising the question of how they escape interferon induction when infecting a mammalian host cell. The answer may lie in a 5'-hairpin structure at the 5'-end of the viral mRNA which prevents detection by RIG-like sensors.
Last of the four pathways, viruses from the Arenaviridae, Bunyaviridae and Orthomyxoviridae families use a ‘cap snatching’ strategy: they steal the cap structure from cellular mRNA (Figure 2c). For this purpose, a cap-binding domain of the polymerase (or N protein) first recognizes the 5’-methylated cap-1 of host mRNAs. In Orthomyxoviridae and Bunyaviridae, the cellular mRNA is cleaved 10–20 nucleotides downstream from the cap structure by a viral endonuclease [19,20,21,22]. The snatched RNA is shorter (4–7 nucleotides) for the Arena-
viridae endonuclease [23]. These short-capped RNAs are subsequently used as primers for viral mRNA synthesis by the viral polymerase. By using this strategy, viruses kill two birds with one stone: de-capping of cellular mRNA blocks the expression of cellular RNA while favoring the expression of viral RNAs.

**Enzymes involved in viral RNA capping pathways**

**5'-RNA triphosphatase**

When nascent viral RNA emerges from the viral replicase/transcriptase, the 5’-pppRNA is processed to 5’-ppRNA before being decorated with the guanine cap. There are five types of viral 5’-RNA triphosphatases involved in this first step of the canonical viral RNA capping pathway.

Metazoan metal-independent RTPases, such as that of the baculovirus BVP, constitute the first type [24]. These enzymes belong to the cysteinyl-phosphatase family, whose fold and catalytic mechanism have a large number of cellular counterparts, thus limiting its interest as a drug design target for the sake of selectivity.

In the second type, hydrolysis of the RNA 5'-γ-phosphate is achieved by genuine metal-dependent dedicated viral 5'-TPase. This is the case in plant, fungi, protozoans, and DNA viruses (mimivirus, poxviruses, baculoviruses have 2 distinct TPases), with the so-called Triphosphatase Tunnel Metallo-enzymes (TTM) superfamily [11,25]. The metal-dependent active-site lies at the bottom of a tunnel, the shape of which seems well-suited to accommodate specific inhibitors exhibiting binding affinities in the nanomolar range [26]. The third and fourth type are those of Reoviridae, which also have their genuine 5’-RTPase: the HIT-like family of Rotavirus, an octamer of the NSP2, and the so-called RNA cap assembly line, a large enzyme complex encompassing VP4 and α2 of Bluetongue and mammalian orthoreovirus, respectively [27,28]. The RNA cap assembly line represents a model of concealing nascent viral RNA and compaction of a chemical reaction sequence. In the last, fifth type, hydrolysis of the RNA 5’-γ-phosphate is achieved by the ‘engine’ of the viral helicase whose NTPase active site is also able to accommodate the 5’-pppRNA [29]. This NTPase active site incorporates a DEAD/H sequence (Walker B motif) and is responsible for fueling the helicase movement along RNA. An inhibitor at this site should therefore be bi-functional, killing both helicase and RTPase/capping activities. However, probably due to the highly dynamic nature of the helicase enzyme, few potent inhibitors have been reported so far [30].

**Guanylyltransferase**

In the viral RNA capping pathway, GTases are amongst the first enzymes to have been identified nearly forty years ago, as they form an easily detectable GMP-enzyme adduct [7–11]. Biochemical and structural characterization has revealed that GTases belong to the ATP-dependent DNA ligase family [31]. However, only few virus families (some dsDNA viruses and Reoviridae) rely on these ‘pure’ GTases included into the capping pathway between RTPases and RNA cap-MTases. GMP is loaded onto the ε-NH₂ of a catalytic lysine part of a KXDG(1/L) motif to form the typical covalent adduct, which is later transferred to the viral 5’-diphosphate RNA. A detailed mechanism has been inferred from elegant crystallographic and mechanistic studies [11]. Few GTase inhibitors have been isolated yet (Figure 3). Mechanistic data might serve to guide the design of active-site inhibitors, provided that selectivity is achievable amongst the large number of cellular GMP-transfer enzymes.

Remarkably, many other RNA viruses use variations of this ping-pong mechanism of GMP transfer (e.g., see NNS viruses and Topacaviridae below). Details of guanine-cap acquisition also remain elusive for the large flavivirus genus, as well as the whole Nidovirales order (Coronaviridae, Arteriviridae, etc). In flaviviruses, a putative covalent GMP-MTase domain adduct has been proposed. Since the putative catalytic lysine with its ε-NH₂ is not conserved, the covalent adduct to NS5 remains questionable. For flaviviruses (and perhaps Nidovirales), it may well be that specific 5’-RNA sequences, which are required to express the N7-MTase, are essential elements to catalyze cap addition without any covalent intermediate.

**Cap-methyltransferase**

The methylation of the cap involves two activities: the N7-MTase that transfers a methyl group on the cap guanosine residue and the RNA 2’-OMTase which methylates the ribose of the N1 residue of the cap structure. Despite their highly divergent sequences, most MTases harbor a canonical Rossmann fold with a β-adenosyl methionine (SAM, methyl donor) binding pocket, an RNA- and/or cap-binding site and a catalytic site [32]. The SAM is usually maintained in close proximity of the catalytic site by a conserved D-X-G motif. The 2’O MTases have been shown to harbor a conserved K-D-K-E catalytic tetrad, which participates to substrate positioning for an in-line SN2 methyl transfer reaction where the methyl on the sulfur atom of SAM
Structure of inhibitors targeting enzymes involved in viral RNA capping pathways. Few inhibitors blocking the GTase activity have been reported: Ribavirin 5′-triphosphate was first proposed to target the GTase activity of capping enzymes [55]. Screening efforts have identified thioxothiazolidin and MADTP derivative as potent inhibitors of flaviviruses and chikungunya virus GTase [45*,57]. Several MTase inhibitors have been reported: Ribavirin 5′-triphosphate ([58], reported in the GTase panel) and the SAM/SAH analogue Sinefungin are inhibitors of several viral MTases in vitro and SAH analogues derivative (compound 10) also inhibit more specifically the flaviviral MTase [38]. Non-nucleosidic inhibitors, obtained by a fragment-based drug-design approach targeting the flaviviral MTase (Dengue and Zika virus) have also been reported recently [39,59]. Virtual screening was also used to identify dengue MTase inhibitors (NSC series [60]) against Zika virus (F3043-0013 and F0922-0796) [40]. Compounds regulating the SAH/SAM balance such as 3-deazaneplanocine A show potent broad-spectrum antiviral activity, including Ebola virus (see text). This antiviral effect is supposedly linked to inhibition of their MTase through increase of the SAH pool. The chemical structures of representative EndoN inhibitors are shown; most of them have been crystallized within the active site of the influenza virus PA enzyme [51]. Cap analogues exemplified here with 3′GTP, and several inhibitors of cap-binding protein have been identified through X-ray structure analysis of the influenza virus PB2-CBD in complex with the corresponding ligands. For RO0794238, direct binding to the PB2-CBD could not be demonstrated [52]. The VX-787 corresponds to a highly potent Influenza PB2 inhibitor [53] 1.

serves as the electrophile that undergoes attack by the activated 2′ oxygen of the RNA substrate [33]. SAM is converted to S-adenosyl-L-homocysteine (SAH) during this process. In contrast, the N7-MTase mechanism remains elusive as catalysis does not involve a tetrad of residues easily identified in protein sequences. Some virus families code for two different MTase domains carrying a cap-binding site (e.g., poxviruses [11], coronaviruses [14,34,35]) involved in N7- or 2′O-methylation of the cap structure. In contrast, other virus groups (e.g., flaviviruses, *Mononegavirales*) have evolved one MTase domain harboring both N7- and 2′O-MTase activities [12,15,36**]. Cap methylation thus implies a step of RNA repositioning allowing the presentation of either the guanine N7 position or the 2′O position of the first transcribed nucleotide in close vicinity to the SAM methyl donor. As the N7 methylation is essential for viral mRNA translation into protein and the cap 2′O
methylolation limits viral RNA detection by RIG-like sensors, such MTases bear potential as new antiviral targets [37**].

A first class of inhibitors consist of SAM-mimetics acting as competitors against the MTase co-substrate. Whereas most viral MTases can be inhibited by SAM analogues such as sinefungin, it is likely that more specific inhibitors can be discovered upon structure–activity relationship analysis (Figure 3). Accordingly, some SAM mimetics accommodating specifically an unique hydrophobic pocket adjacent to the SAM-binding site in flavivirus MTases inhibit Dengue virus replication [38]. In addition, docking experiments indicate that those compounds might bind to the highly homologous MTase of Zika virus (ZIKV) [39]. A second possibility to develop specific inhibitors is illustrated by fragment-based drug design approaches. Using this approach, allosteric inhibitors targeting DENV and ZIKV MTases in vitro were recently reported, but their median inhibitory activity remain in the 10–50 μM range [39]. Additionally, using a virtual screening approach, ten potential inhibitors of the ZIKV MTase were sorted out of 28341 compounds. Even if these compounds were not yet demonstrated to limit the MTase activity in vitro, they show antiviral activity in ZIKV-infected cells with EC_{50} ranging from 4 to 17 μM. Thus it is likely that molecular docking can be used to target conserved ‘druggable’ sites and design-specific MTase inhibitors [40]. A third possibility has emerged with MTases activated by a protein partner (e.g., corona-viruses), for which peptidometimics have been reported as specific inhibitors [41]. Finally, it is also possible to down-regulate the MTase activity level by modulating the intracellular SAM/SAH balance using 3-deazaadenosine, an SAH hydrolase inhibitor [42]. This strategy was successfully used to inhibit Ebola virus replication in mice, although the latter inhibitor might also have an effect on interferon production [43,44].

The togaviridae/alphavirus GTase/MTase
Alphaviruses have evolved a bi-functional MTase/GTase embedded into the nsP1 protein that synthetizes a cap-0 structure. In contrast to most viral MTases, nsP1 uses GTP rather than a cap structure as a substrate. The GTP is thus first methylated at its N7 position (m^{7}GTP). The m^{7}GTP then forms a covalent link (m^{7}GMP-nsP1) with the catalytic histidine of nsP1, releasing inorganic pyrophosphate [16,17,18*] (Figure 2b). The methylated GMP is subsequently transferred onto the nascent viral RNA yielding a cap-0 structure. Although no structural data are yet available for any nsP1, the uniqueness of this unconventional capping reaction makes it an attractive target for antiviral drug design. Recently small molecule inhibitors of alphavirus nsP1 were reported to block CHIKV replication in the μM range (2–26 μM, Figure 3) [45**]. By selecting resistant viruses, it was demonstrated that the guanylyltransferase activity was the target.

The NNS GDPase/PRNTase
This enzyme performs RNA cap-0 addition through a mechanistic variation of the classic RTPase/GTase pathway. Whereas GTases bind GMP covalently and accept a 5’-diphosphate RNA, PRNTases bind 5’-monophosphate RNA and accept GDP (Figure 2a). Both pathways lead to the same end product, the RNA cap-0. One could think that the chemistry underlying covalent attachment of GMP or pRNA would be related, since GTP and 5’-triphosphate RNA are structurally related and both receive a nucleophilic attack at their α-phosphate. However, GTases bind GMP through a ε-lysyl-phosphoramidate intermediate [10], whereas PRNTases bind 5’-monophosphate RNA through a N^{ε2}-histidine intermediate [46]. Furthermore, the protein folding of GTases and PRNTases does not show any obvious homology, suggesting that this covalent attachment promoting a ping-pong mechanism has been invented twice during evolution. From the drug design point of view, GTases might be difficult to inhibit with high selectivity. Indeed, they belong to the large family of DNA ligases [31], whose structure and activity is largely represented in the mammalian world. PRNTases are uniquely represented in NNS viruses. The activity has been demonstrated in Rhabdocoelidae only so far, but PRNTase-like domains have been identified in many other NNS virus families such as Paramyxoviridae, Pneumoviridae, Filoviridae, to name a few families of significant medical interest. Recent progress in structural determination of these enzymes at atomic resolution [47**] should greatly stimulate drug design in the near future.

Endonucleases in cap snatching
Three viral families (Orthomyxoviridae, Arenaviridae, and Bunyaviridae) carry a cap-binding domain and an RNA endonuclease domain in their replicase complex (see above). Whether the latter domain is fused to the polymerase core or carried on a separate subunit, the folding and mechanism of action of the endonuclease is the same, suggesting a common phylogenetic origin. The endonuclease belongs to the PD-D/Eκ superfamily of cation-dependent nucleases, in which lysine is the main catalytic residue. The acidic D and D/E residues coordinate two metal ions, together with a histidine residue conserved in Orthomyxoviridae and Bunyaviridae enzymes, or replaced by a third acidic residue in Arenaviridae [22**]. All these endonucleases share a common two-metal-ions mechanism, with distinct metal preferences though, and this feature can be exploited for drug design as the active site environment shows some virus-specific structural variation. Metal chelators have shown their ‘druggability’, such as raltegravir and related pharmacophores, in the case of inhibition of the HIV integrase. It is indeed possible to chelate the metals at the enzyme active site while obtaining target specificity through occupancy of the unique active site environment [48]. Hence, metal chelators, such as 2,4-dioxo-4-phenylbutanoic acid (DPBA) that acts
as bunyavirus and orthomyxovirus endonuclease inhibitor, may provide a pharmacophoric motif to design more potent compounds.

The cap-binding domain is also an attractive antiviral target (Figure 3). The X-ray structure of influenza A or B virus PB2 in complex with m<sub>7</sub>GTP [49,50] reveals a conserved cap-recognition mechanism in which the methylated guanosine is stacked between two aromatic residues similar to its binding mode with the eukaryotic initiation factor (eIF4E). However, the PB2 folding is unique compared to other cap-binding proteins, raising the possibility to identify specific inhibitors [51]. Cap analogue pharmacophores were first demonstrated to selectively inhibit PB2 cap-binding, with no effect on eIF4E [52]. More recently, VX787, another PB2 cap-binding domain blocker for influenza virus, was identified [53]. This compound shows robust antiviral activity and is now in phase 2 trial demonstrating the validity of PB2 as antiviral target.

**Biochemical principles and inhibitors to interfere with viral RNA capping**

RNA capping reactions involve a number of different enzymes and RNA substrates in order to make a *bona fide* RNA cap. The efficiency of an inhibitor will depend on biochemical properties, embedded in its chemical structure, such as ability to access to replication sites, to bind to the viral target, to interfere at the catalytic step, or to impede enzymatic conformational change(s). Figure 3 reports the main inhibitors having shown anti-RNA capping properties.

**Binding step**

A number of different binding sites on RNA capping enzymes accommodating those substrates represent natural targets for inhibitors. These substrates exhibit a wide chemical diversity, from small organic molecules, for example, S'-adenosyl methionine to large polar substrates, for example, RNA. Aiming at competitive inhibitors displacing those substrates will have to take into account mainly two factors: the substrate binding energy (binding surface, strength of bonding), which could be high in the case of a large RNA binding groove, and the actual intracellular concentration of the natural, competed substrate. In the case of GTP, a putative competitive inhibitor would have to reach high intracellular concentrations and/or exhibit a very high affinity to displace millimolar concentrations of this natural nucleotide. For analogues competing with SAM or SAH, cellular concentrations of the latter are about an order of magnitude lower [54].

**Catalytic step**

Inhibition does not depend on substrate binding affinity only, but also on the efficiency of the following catalytic reaction. For example, Ribavirin S'-TP is an RNA capping inhibitor when it makes a Ribavirin S'-MP covalent adduct on the GTase. Its inhibition efficiency depends thus on how tightly Ribavirin S'-triphosphate binds, but also how fast it reacts to be covalently linked, and thus will be given by a ratio of the catalytic constant k<sub>cat</sub> divided by the affinity constant K<sub>d</sub>. Detailed kinetic constants obtained using pre-steady state kinetics are still lacking to describe precisely the different RNA capping pathways.

**Indirect effects**

Other possible mechanisms of action of an RNA capping inhibitor are, e.g. its direct action on the intracellular pool of RNA capping substrates. This is exemplified by Ribavirin, whose mechanisms of action is clearly pleiotropic [55,56], and whose metabolite Ribavirin S'-monophosphate is known to inhibit the cellular IMP dehydrogenase enzyme, resulting in a profound depletion of the intracellular GTP pool. This depletion has a pleiotropic effect into which stands inhibition of the GTase reaction. Another example is 3-deazaneplanocin A, which is an inhibitor of the cellular SAH hydrolase. The latter enzyme maintains a low level of SAH, which is a potent reaction-product inhibitor of the RNA cap-MTase [42].

**Protein–protein interaction and conformational changes**

Structural data on large replicase/transcriptase complexes, such as those of NNS viruses, *Orthomyxoviridae, Bunyaviridae*, have emerged recently and shown that replicase/transcriptase complexes perform RNA synthesis and capping in a concerted manner, with RNA and substrates traveling in between several protein domains. It is thus likely that allosteric inhibitors will be found to act as 'sand in the machine', and alter the finely tuned conformational changes and structure re-arrangements needed to expedite RNA capping events. It is also possible that protein–protein interfaces will be targets of choice in the near future to design entirely novel classes of antivirals. As SAM is used as methyl donor by many cellular MTases it is likely that the development of allosteric or protein–protein interaction inhibitors may be superior to SAM mimetics or SAH hydrolase inhibitors, in terms of selectivity, when designed toward a unique site in the viral enzyme.

**Conclusions**

Viral RNA capping is taking place at each genome replication event in (+)RNA viruses (*e.g.*, flaviviruses), and to a much higher occurrence when several viral mRNAs are generated for each replicated genome (*e.g.*, NNS viruses, *Nidovirales*, . . .). The significance of RNA capping as an antiviral target could legitimately be questioned in some instances, for example, when RNA capping is a rare event during the virus life cycle. However, the past research decade has *a contrario* unveiled that RNA capping is essential for virus replication, and is in fact a most interesting target for the design of potent antivirals due to two main reasons: (i) incomplete/inhibited RNA
capping triggers a potent host immune response adding up to direct inhibition of viral gene expression, and (ii) structural and functional studies of viral capping enzymes have revealed a profound uniqueness of the viral enzymes involved, which shows promises to achieve high drug selectivity. There is thus little doubt that high resolution structures coupled to detailed enzyme mechanisms will inspire design of highly potent direct-acting antivirals in the near future.

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