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# Cholesterol-conjugated peptide antivirals: a path to a rapid response to emerging viral diseases‡

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**While it is now possible to identify and genetically fingerprint the causative agents of emerging viral diseases, often with extraordinary speed, suitable therapies cannot be developed with equivalent speed, because drug discovery requires information that goes beyond knowledge of the viral genome. Peptides, however, may represent a special opportunity.**

**For all enveloped viruses, fusion between the viral and the target cell membrane is an obligatory step of the life cycle. Class I fusion proteins harbor regions with a repeating pattern of amino acids, the heptad repeats (HRs), that play a key role in fusion, and HR-derived peptides such as enfuvirtide, in clinical use for HIV, can block the process. Because of their characteristic sequence pattern, HRs are easily identified in the genome by means of computer programs, providing the sequence of candidate peptide inhibitors directly from genomic information.**

**Moreover, a simple chemical modification, the attachment of a cholesterol group, can dramatically increase the antiviral potency of HR-derived inhibitors and simultaneously improve their pharmacokinetics. Further enhancement can be provided by dimerization of the cholesterol-conjugated peptide. The examples reported so far include inhibitors of retroviruses, paramyxoviruses, orthomyxoviruses, henipaviruses, coronaviruses, and filoviruses. For some of these viruses, *in vivo* efficacy has been demonstrated in suitable animal models.**

**The combination of bioinformatic lead identification and potency/pharmacokinetics improvement provided by cholesterol conjugation may form the basis for a rapid response strategy, where development of an emergency cholesterol-conjugated therapeutic would immediately follow the availability of the genetic information of a new enveloped virus. Copyright © 2014 European Peptide Society and John Wiley & Sons, Ltd.**

**Keywords:** emerging viral disease; emerging virus; bioterrorism; emergency and preparedness response; viral fusion; fusion inhibitor; cholesterol conjugation; dimerization; peptide antiviral

## The Threat of Emerging Viral Diseases

The spectrum of infectious diseases that threaten human health is rapidly broadening: The number of previously unknown conditions that have emerged since 1970 exceeds 40, with a new disease discovered on average more than once a year [1,2]. According to a 2008 World Health Organization (WHO) report, between the years 2002 and 2007, a record of 1100 worldwide epidemic events occurred [3]. A comprehensive examination of episodes of emerging infectious disease outbreaks between 1940 and 2004, after controlling for reporting bias, came to the conclusion that the incidence of the events has increased significantly over time [4].

For viral diseases in particular, in addition to the constant fear of a new wave of pandemic influenza, the list of emerging or reemerging viruses includes HIV, hantavirus, Lassa virus, Marburg virus, hepatitis C virus, dengue virus, Rift Valley fever virus, Ebola virus, Nipah virus, Hendra virus, West Nile virus, severe acute respiratory syndrome (SARS) coronavirus, avian influenza virus, human polyomavirus, adenovirus 14, Chikungunya virus, and most recently the Middle East respiratory syndrome coronavirus [5]. At the time when this manuscript was being finalized, the largest and longest outbreak of Ebola virus was still spreading [6]. The latter virus, together with Nipah virus, hantavirus, Lassa virus, and Marburg virus, is on the Centers for Disease Control and Prevention list of bioterrorism agents. For most of these viruses, the therapeutic options are limited if not completely absent.

## Prevention and Therapy of Emerging Viral Diseases

The best description of the features of an outbreak of a novel virus comes from the SARS pandemic of 2002/2003 – the first major emerging disease threat of the 21st century. In the short period between the WHO global alert (March 2003) and the WHO announcement that the pandemic was over (5 July 2003), SARS affected 8098 people, caused 774 deaths, disrupted international travel, and cost huge business losses. SARS presented many of the features of the most feared pandemic diseases: a respiratory disease that is transmitted from person to person, with a long asymptomatic incubation period, and symptoms very similar to other commonplace illnesses.

At the time of its first appearance, its causative agent (SARS-associated coronavirus) was unknown, and there was neither a diagnostic test nor a specific treatment. The international response to the

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**Biography**

Antonello Pessi obtained his Chemistry Laurea degree at the University of Rome. After a short stay in the laboratory of R. Sheppard at LMB (Cambridge, UK), he held positions of increasing responsibility at ENIRICERCHE, SCLAVO (now part of GSK), and IRBM, the Italian site of Merck Research Laboratories, where he spent 20 years. Under his direction, the Peptide Centre of Excellence, the centralized Merck Unit for peptide medicinal chemistry, provided a number of preclinical candidates to the company pipeline. In 2009, he founded PeptiPharma, which offers consulting and contract research on therapeutic peptides. He is also the CSO of JV Bio, a start-up focused on clinical development of antiviral peptides and antibodies. He is the recipient of the 2005 Leonidas Zervas Award of the European Peptide Society and author of more than 160 publications and 40 patents. His current academic interest, in collaboration with CEINGE, a nonprofit consortium of the University of Naples, is the design of antibacterial peptides.

new threat was extremely efficacious, leading to the isolation of SARS-CoV [7] and sequencing of its genome in the record time of just 2 weeks [8,9].

The measures that proved efficacious in containing the disease were isolation, quarantine, travel surveillance, increased personal hygiene, and personal protection equipment (masks, gloves, and eyeglasses). As to pharmacological treatments, however, a WHO expert panel review concluded that it was not possible to determine whether any of the treatments used for SARS-infected patients did some benefit and suggested that some treatments might have actually been harmful [10].

As the SARS case vividly illustrates, our ability to quickly isolate and genetically fingerprint the causative agent of new viral diseases is not matched by an ability to develop suitable treatments. This is not surprising, because drug discovery typically requires additional information to that simply deriving from knowledge of the viral genome.

## A Role for Peptides: Inhibition of Viral Fusion

Against this background, peptides may represent a special case, because for enveloped viruses, it is generally possible to identify the sequence of candidate peptide inhibitors directly from genomic information.

For all enveloped viruses, fusion between the viral and the target cell membrane represents an obligatory step of the life cycle. Interfering with this process is a well-established therapeutic strategy [11] that has led to the development of the peptide fusion inhibitor enfuvirtide (Fuzeon®, also known as T20, Roche, Basel, Switzerland), which is in clinical use for HIV [12].

Viral fusion is mediated by specialized proteins, which harbor a structural motif that consists of a repeating pattern of seven amino acids: the heptad repeat (HR). The most prevalent class (class I) of fusion proteins typically has two HR regions: the first one close to the N-terminus (HRN), adjacent to the fusion peptide, and the second one close to the C-terminus (HRC), immediately preceding the transmembrane domain. It is generally accepted that in the key intermediate of viral fusion, the so-called prehairpin intermediate, bridging the viral and cell membranes, the HRN and HRC regions are separated, and the HRN forms a trimeric coiled coil (Figure 1). Folding of the HRC onto the HRN trimer leads to the formation of a six-helical bundle (6HB), and in this process, the two membranes are brought in close apposition, which leads to their fusion [11] (Figure 1). Peptides corresponding to the sequence of the HR regions can bind to the prehairpin intermediate, prevent its transition to the 6HB, and block fusion (Figure 2(A)). This is the

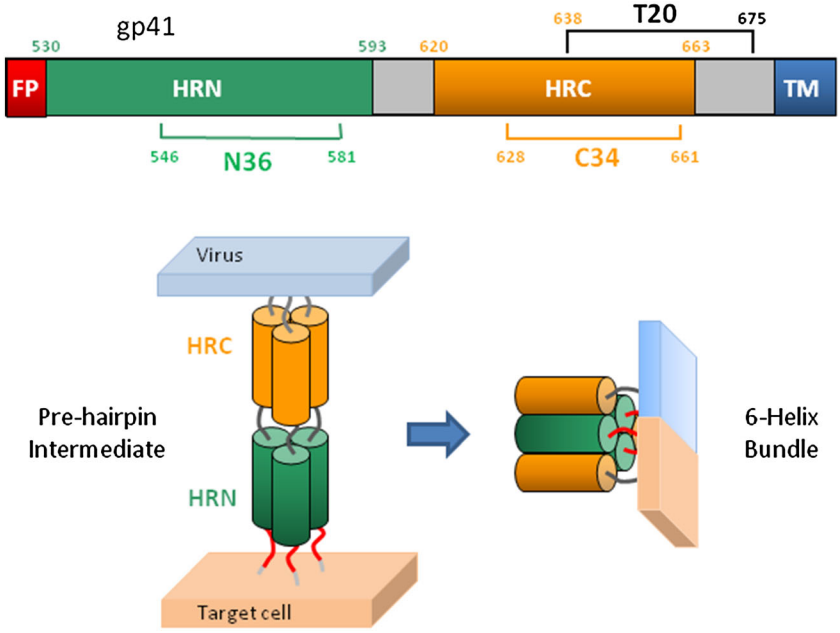


**Figure 1.** Viral fusion, exemplified by HIV. The fusion protein of HIV, gp41, is schematically drawn at the top of the figure. Gp41 features two heptad repeat (HR) regions, which are separated in the prehairpin intermediate. The fusion peptide (FP), N-terminal to the HRN, anchors the protein into the target cell membrane. The protein is also anchored in the viral membrane by the transmembrane (TM) region, C-terminal to the HRC. Refolding of the intermediate leads to formation of a six-helix bundle (6HB), bringing the two membranes in close contact and driving fusion.

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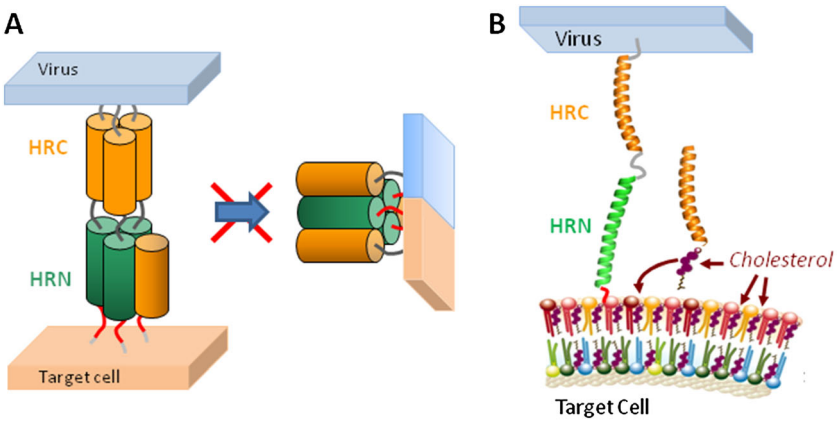


**Figure 2.** Inhibition of viral fusion. (A) HR-derived peptides such as C34 block fusion by binding to the prehairpin intermediate and preventing the intramolecular refolding to the 6HB. (B) A cholesterol-conjugated HR-derived peptide concentrates in the cholesterol-rich membrane domains, which are the sites where fusion occurs.

mechanism of inhibition of enfuvirtide [12], which applies to several other peptides derived from the HR regions of many viruses [13–29].

## Prediction of Fusion Inhibitors from the Viral Genome

The characteristic sequence pattern of the HR provides an important advantage for the development of peptide-based antivirals, because HR can be easily identified through computer programs (e.g. LearnCoil [30] or MultiCoil [31,32]). This was the case for SARS-CoV, where HR regions were immediately identified [33] and led to the development of peptide inhibitors [27,29], in the absence of any structural information on the fusion protein. This came later and largely confirmed the predictions [34–37].

## From Fusion Inhibitors to Antiviral Drugs

This pathway to drug development offers the opportunity to develop a *specific* antiviral in a very short timeframe. However, not all HR-derived peptides display the same antiviral potency as the HIV fusion inhibitor enfuvirtide. A major factor at play is the difference in fusion kinetics: viruses that unlike HIV transition through the hairpin intermediate very rapidly offer a shorter window of opportunity for peptide inhibitors that, accordingly, show reduced potency [38–40].

If the peptide lead derived from genomic information has insufficient potency, it is necessary to modify it through peptide engineering strategies that, although often successful [18,41–49], may be time consuming. An alternative approach, which does not require any change in the native HR sequence, exploits the role of cholesterol in viral fusion.

## Role of Cholesterol in Viral Fusion

Cholesterol plays a key role in the fusion and budding of enveloped viruses [50–59], with HIV as the most studied case. It has been shown that cholesterol depletion from virus-infected cells suppresses virus production [60] and that depletion of cholesterol from either virions or target cells inhibits virus–cell fusion [61–64]. HIV is able to influence the cholesterol metabolism of the host cell [65–68], and its lipid membrane is highly enriched in cholesterol [69]. Moreover, a link has been established between low levels of cholesterol in antigen-presenting cells and the status of long-term nonprogressor, a rare infected individual who controls disease progression in the absence of antiretroviral therapy [70,71].

The role of cholesterol in viral fusion is related to its role in membrane fluidity and in particular in the formation of lipid rafts [60,72–76]. Accordingly, the composition of the lipid membrane of HIV is highly enriched in cholesterol and sphingomyelin [69,74,75]. Moreover, CD4, the primary receptor for HIV, is enriched in lipid rafts [77].

### Exploiting cholesterol conjugation for fusion inhibitor design

In 2009, Ingallinella *et al.* proposed that addition of a cholesterol group to an HR-derived peptide would augment its affinity for membranes and in particular for the cholesterol-rich lipid rafts. The resulting increase in local concentration at the site of action would translate into enhanced antiviral potency (Figure 2(B)). Application of this concept to the HR-derived peptide C34 yielded C34-Chol, where the cholesterol group was attached, via a thioether bond, to the side chain of a Cys residue added to the C-terminus, with an intervening Gly–Ser–Gly spacer. Cholesterol conjugation brought a dramatic improvement of antiviral potency: Depending on the viral strain tested, C34-Chol was 25- to 100-fold more potent than unconjugated C34 and 50- to 400-fold more potent than enfuvirtide [78]. No such improvement was apparent when cholesterol was substituted with palmitic acid [78], a lipid that is both a poorer membrane anchor and is not enriched in lipid rafts [79].

**Table 1.** Viruses for which cholesterol conjugation increases the antiviral potency of a heptad repeat-derived peptide inhibitor

| Virus | Reference |
|---|---|
| HIV | [78,80,93,95] |
| Parainfluenza virus | [93,111] |
| Influenza virus | [112] |
| Nipah virus | [86,93,111] |
| Hendra virus | [111] |
| SV5 (HPIV5) | [111] |
| Measles virus | [94] |
| Ebola virus | [113] |
| Newcastle disease virus | [87] |
| Infectious bronchitis virus | [87] |



  

Numerous examples have since accumulated that cholesterol conjugation can improve the antiviral potency of HR-derived peptides from many enveloped viruses – these include *in vitro* and *in vivo* studies (Table 1). Cholesterol conjugation has also been successfully applied to a covalent inhibitor derived from C34, where an isothiocyanate group on the side chain of $Asp^{632}$ targets $Lys^{574}$ in HRN [80].

Interestingly, in line with the hypothesized mechanism of action of cholesterol conjugation, another lipid that is enriched in the HIV lipidome, dihydrosphingosine [69], also augments potency when conjugated to an HR peptide; in this case, one derived from the HRN instead of the HRC [81].

### Cholesterol-conjugated peptides show improved pharmacokinetics and are active *in vivo*

Cholesterol conjugation also solves an outstanding problem of peptide therapeutics, the short *in vivo* half-life because of enzyme degradation and rapid clearance [82]. An effective way to improve peptide pharmacokinetics (PK) is conjugation to lipids [82], which drive binding to serum proteins. The most typical derivatization is with long chain fatty acids, but derivatization with cholesterol has also been explored [83–85].

In a comparative subcutaneous PK study in mice at the concentration of 3.5 mg/kg, C34 was undetectable in plasma after 6 h, while 130 nM of C34-Chol was still detectable 24 h after the injection: a concentration ≈300-fold higher than the $IC_{90}$ measured against multiple HIV-1 strains [78].

In another example, a single dose of 2 mg/kg of a cholesterol-conjugated inhibitor of Nipah virus, administered intraperitoneally to golden hamsters, was sufficient to achieve a plasma concentration >100-fold higher than the *in vitro* $IC_{50}$ for 24 h [86].

A single intramuscular injection of 1.6 mg/kg of a cholesterol-conjugated inhibitor of Newcastle disease virus (NDV) administered to chickens yielded a plasma concentration of 210 nM at 24 h and ≈120 nM after 48 h, to be compared with the *in vitro* $IC_{50} = 8.1$ nM and $IC_{90} = 13$ nM [87].

Overall, the PK of a cholesterol-conjugated inhibitor is expected to be at least comparable with – and likely better than – the PK of enfuvirtide, which is administered twice daily by subcutaneous injection [88].

### Dimerization to complement cholesterol conjugation

One further possibility to potentiate the activity of HR-derived peptide inhibitors is to exploit multimerization, another modification that can be implemented on the native sequence of the peptide. Multimerization brings an avidity effect [89], which effectively reduces the $k_{off}$ of the inhibitor fusion protein complex and increases the potency of fusion [90,91] and entry [92] inhibitors. The effect of cholesterol conjugation, which increases the $k_{on}$ by facilitating the encounter of the peptide with the viral target protein, is complementary: The two modifications can therefore work in concert.

A reagent that can simultaneously dimerize the peptide and install a cholesterol group onto it is described in Figure 3 [93]. Trimers or higher-order multimers could be prepared by the same strategy, but the maximum benefit of multimerization is achieved at the level of a dimer [90,91]. The reagent is stable upon prolonged storage at −20 °C. Notably, the same cysteine-containing precursor peptide used to produce the monomeric cholesterol-conjugated peptide can be reacted with the cholesterol dimer-forming moiety: This enables parallel synthesis of the cholesterol-conjugated monomer and dimer (Figure 3), which can then also be tested in parallel to establish the optimal inhibitor.

Using the reagent in Figure 3, dimeric cholesterol-conjugated inhibitors were prepared for HIV [93], Nipah [93], and measles [94] virus (MV), which were more potent and effective than the corresponding cholesterol-conjugated monomers. Potentiation of a trimeric HIV peptide inhibitor [91] by cholesterol conjugation [95] has independently been reported by another laboratory.

## Scope of Cholesterol Conjugation

Since its discovery in 2009, the scope of cholesterol conjugation has considerably increased, and it now includes examples for retroviruses, paramyxoviruses, coronaviruses, orthomyxoviruses, henipaviruses, and filoviruses (Table 1).

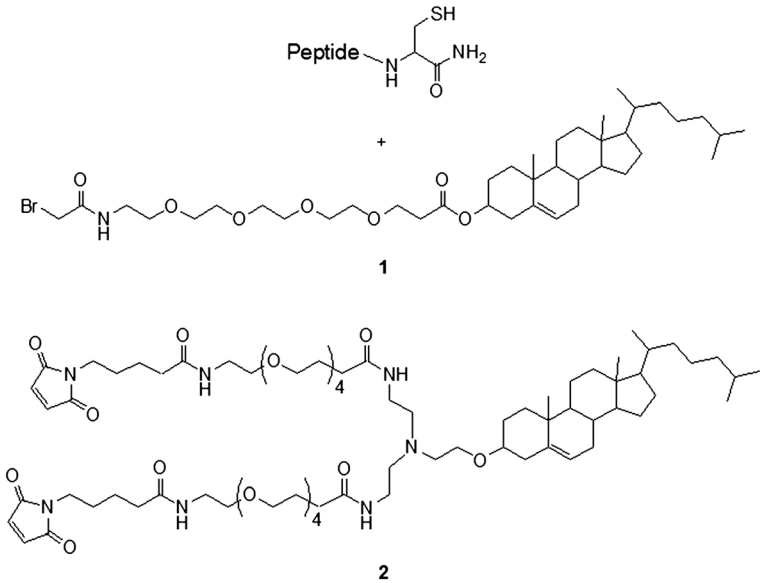


**Figure 3.** Parallel cholesterol conjugation. Reaction of the same peptide precursor with off-the-shelf reagents **1** and **2** yields the monomeric and dimeric cholesterol-conjugated inhibitors. The bromoacetyl functionality was unsuitable for reagent **2** [93].

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For some of these viruses, *in vivo* efficacy has been demonstrated in a suitable animal model [86,87,94]. For example, Porotto *et al.* showed that once-daily intraperitoneal administration of a cholesterol-conjugated inhibitor of Nipah virus to golden hamsters, an established model of infection, effectively prevented what would otherwise be fatal Nipah virus encephalitis. Prophylactic treatment was 100% efficacious when initiated 2 days before viral challenge and 80% efficacious when initiated concurrently with the infection. Notably, in light of the highly lethal nature of this virus, treatment 2 days after infection led to survival of 40% of the animals [86].

The same laboratory used intranasal infection of MV in suckling mice expressing the human SLAM transgene as a model to evaluate the efficacy of cholesterol-conjugated HR-derived peptides. Infection with MV causes a lethal acute neurological syndrome, which was completely prevented, in 100% of the animals, by prophylactic once-daily administration of a cholesterol-conjugated dimer derived from the sequence of the MV HRC domain [94].

Li *et al.* used cholesterol conjugation to improve the potency of their previously described inhibitor of NDV [96]. Intramuscular injection of the cholesterol-conjugated peptide 1 day before virus infection, and then every 3 days, protected 70% of the chickens from different serotypes of NDV [87]. Moreover, treatment of the animals 2 days after infection resulted in 50% protection [87].

Two interesting features of these studies deserve further comment: First, in the aforementioned three studies, the peptide was detectable in the brain 24 h after administration [86,87,94], indicating that cholesterol conjugation may enable penetration of the blood–brain barrier, a difficult feat for drugs in general and for biologics in particular [97].

Second, the modest but observable degree of protection observed at 2 days postexposure (40% in the Porotto *et al.* [86] and 50% in the Li *et al.* [87] study) is comparable with the results reported for monoclonal antibodies against Ebola virus in macaques (50% efficacy when dosed 2 days after exposure) [98,99], including the ZMapp antibody cocktail [99], which is being used as an emergency therapeutic in the latest Ebola outbreak [100].

It must be noted though that all the examples reported so far come from viruses featuring class I fusion proteins. While being the most prevalent class, it does not include major pathogens such as hepatitis C virus and dengue virus, both belonging to class II, and herpes simplex virus belonging to class III.

Class I proteins are mainly $\alpha$-helical and form 6HBs as the postfusion structure, as exemplified by HIV (Figure 1). Class II fusion proteins are characterized by trimers of hairpins composed of $\beta$-structures [101], with a fusion loop located at the tip of an elongated $\beta$-sheet, in place of a fusion peptide upstream of an $\alpha$-helix. Class III proteins form trimers of hairpins by combining a central $\alpha$-helical trimeric core, similar to class I proteins, with fusion loops structurally similar to class II proteins [101]. Despite these structural differences, all fusion proteins adopt a hairpin structure [102] that is susceptible to inhibition.

For example, although the entry of herpes viruses (featuring a class III fusion protein) is a complex process, not yet fully clarified, in which multiple glycoproteins are involved, several laboratories have reported that peptides derived from the HR regions of gB and gH of herpes simplex virus type 1, bovine herpes virus type 1, and human cytomegalovirus are able to inhibit infection [103].

For dengue virus, helical domains have been identified in the stem region of E protein (a class II protein) that are critical for virus assembly and entry [104]. Peptide inhibitors derived from this membrane-binding region have been described [105–107], one of which inhibits all four virus serotypes [107]. Moreover, *de novo* computational design of peptide inhibitors has been successful [108,109]. The work by Xu *et al.* in particular [109] indicates how the same computational approach may yield candidate peptide inhibitors for fusion proteins of all classes.

Given the ubiquitous importance of cholesterol-rich lipid rafts in viral fusion, it is expected that cholesterol conjugation may enhance the antiviral potency also for peptides derived from class II and III fusion proteins. However, no data in this regard have yet been reported.

## Perspective: A Rapid Response Strategy to Emerging Viral Diseases

The combination of bioinformatic lead identification (lead ID) and potency/PK improvement provided by cholesterol conjugation may form the basis for a rapid response strategy to emerging viral diseases (Figure 4). The project would begin immediately after the genome of the new pathogen is made available. The lead ID phase would consist in the bioinformatic analysis (e.g. via LearnCoil [30] or MultiCoil [31,32]) of the newly sequenced genome to identify the HR regions; it could last a few days. As discussed in the previous section, the computational identification of inhibitory peptides for viruses utilizing class II or III fusion proteins would be less straightforward, and the number of candidate peptides to be tested likely larger. However, given the high throughput of parallel solid-phase

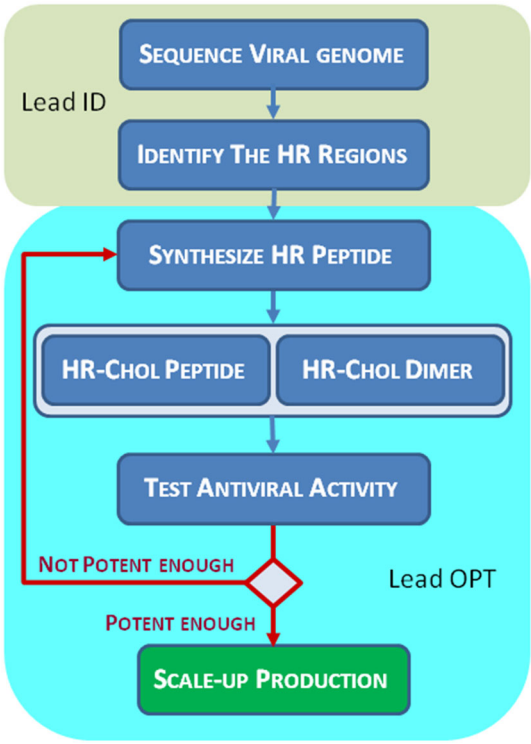


**Figure 4.** Rapid response strategy based on cholesterol-conjugated fusion inhibitors. From the sequenced genome of the emerging virus, the HR regions are identified bioinformatically. This corresponds to the lead identification (lead ID) phase. In the lead optimization (lead opt) phase, candidate HR peptides are synthesized in the form of suitable precursors for cholesterol conjugation and reacted with the reagents shown in Figure 3. The cholesterol-conjugated monomer and dimer are tested for antiviral activity. The lead opt cycle continues until a peptide is found potent enough to be moved to large-scale production.



  

peptide synthesis and the modularity of the cholesterol conjugation reaction, the initial number of target sequences should not represent a key limiting factor in the lead optimization (lead opt) phase. In lead opt, candidate peptides would be synthesized in the form of suitable precursors for cholesterol conjugation and reacted with the reagents shown in Figure 3 or similar ones. The cholesterol-conjugated monomers and dimers would be tested for antiviral activity. In the logic of off-the-shelf reagents for immediate use, variations of the reagents of Figure 3 to optimize the length of the linker joining the monomers might provide considerable increase in potency [89], as shown by Kay *et al*. [91]. Haloacetyl or maleimide-functionalized cores with polyethylene glycol spacers of different length could all be reacted with the same precursor to rapidly select the most potent inhibitor.

The lead opt cycle would continue until a peptide was found, potent enough to be moved to large-scale production. With a relatively small number of candidate peptides, and parallel synthesis and conjugation, it is expected that the lead opt phase would be rapid, optimally a few weeks. Large-scale production of the candidate peptide therapeutic could begin immediately, because the protocol for lab-scale and large-scale solid-phase peptide synthesis are essentially the same. Preclinical evaluation (formulation, animal PK to predict the human dose) could proceed in parallel with the good manufacturing practices batch production. Abbreviated preclinical toxicology, in connection with the 'animal rule' [110], would be justified in an emergency situation.

## Conclusions: A Role for Peptides in the Response to Emerging Viral Diseases

The strategy outlined in the previous section could provide in a very short timeframe a cholesterol-conjugated therapeutic specific for the emerging virus. Although not necessarily the optimal drug for the new disease, it would represent a key complement to preventive measures and could enable better control of the initial outbreak. Further research efforts may then lead to a substitute therapy with increased efficacy and/or better ease of administration.

One could even envisage setting up a core laboratory that routinely runs the lead ID and lead opt stages on newly identified viruses of potential concern, *in advance* of an actual outbreak of disease. The limited resources necessary could be part of the Emergency and Preparedness Response set out by the Centers for Disease Control and Prevention (http://emergency.cdc.gov/) or the Global Alert and response by WHO (http://www.who.int/csr/en/) for preparing for and responding to public health emergencies.

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