Towards antiviral therapies for treating dengue virus infections

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Abstract

Dengue virus is an emerging human pathogen that poses a huge public health burden by infecting annually about 390 million individuals of which a quarter report with clinical manifestations. Although progress has been made in understanding dengue pathogenesis, a licensed vaccine or antiviral therapy against this virus is still lacking. Treatment of patients is confined to symptomatic alleviation and supportive care. The development of dengue therapeutics thus remains of utmost importance. This review focuses on the few molecules that were evaluated in dengue virus-infected patients: balapiravir, chloroquine, lovastatin, prednisolone and celgosivir. The lessons learned from these clinical trials can be very helpful for the design of future trials for the next generation of dengue virus inhibitors.

Introduction

Dengue virus (DENV) infections form a major public health problem in tropical and subtropical areas [1]. The virus belongs to the genus *Flavivirus* within the family of the *Flaviviridae*. Four distinct serotypes have been identified. The main vectors of DENV are *Aedes aegypti*, mainly found in tropical

areas, and *Aedes albopictus*, more common in subtropical areas [1,2]. Once infected, these mosquitoes remain a living reservoir that efficiently transmit the virus. The expanded distribution of these vector species, due to global warming and, for example, international trade in used tires; the failure to control the vectors; and the ongoing urbanization are largely responsible for the dramatic increase of dengue cases worldwide during the last few decades [1,2].

An estimated 390 million people become infected each year, of which about one fourth develops symptoms [3••]. Depending on the geographical region, the mortality rate may vary from 1.2% – 3.5% [1,3••]. Although most DENV infections are asymptomatic or follow a self-limiting non-severe clinical course with undifferentiated fever, dengue fever (DF), a small proportion progresses to potentially life-threatening severe dengue (dengue hemorrhagic fever or dengue shock syndrome). Additional clinical manifestations (typically) include severe headache, retro-orbital pain, muscle and bone/joint pains (hence, the name breakbone fever), nausea, vomiting and rash [1].

The DENV genome is a positive-sense, single-stranded RNA of approximately 11kb, encoding three structural proteins (capsid (C), membrane (M) and envelope (E)), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). Both the structural and non-structural proteins can serve as potential targets for antiviral intervention. Due to the availability of (i) more or less robust cell culture models, (ii) tools for reverse genetics, (iii) crystal structures for the majority of DENV/flavivirus proteins/enzymes, (iv) biochemical assays for various viral enzymes (such as NTPase, RTPase, helicase, protease and polymerase assays [4-9]), and (v) a number of DENV infection models in animals [10-12,13•,14,15], good progress has been made in recent years in developing dengue virus inhibitors. Most of the reported inhibitors target either the NS3 protease, the non-structural protein NS4B, the NS5 RNA-dependent RNA polymerase (RdRP), or the surface glycoprotein E. Clearly, there is a growing interest in developing anti-dengue therapeutics as shown by an increasing number of publications ([16], and reviewed in [17-20,21••,22,23,24•]). Despite these efforts, however, very few compounds are further being developed. This is because of a variety of reasons, including insufficient potency and/or limited pan-serotype activity, poor pharmacokinetic properties and adverse effects in animals.

The potential activity of only a handful of molecules has been evaluated in therapeutic trials. Except for balapiravir (a nucleoside analogue that was initially developed for the treatment of chronic infections with hepatitis C virus and that is also endowed with *in vitro* activity against flaviviruses) all other molecules evaluated so far in such trials are to be classified as non-specific inhibitors, that is chloroquine, lovastatin, prednisolone, and the glycosylation inhibitor celgosivir. These will here be discussed more in detail.

Balapiravir

Balapiravir (R1626; PubChem CID: 11691726), a prodrug of the nucleoside analog 4'-azidocytidine (R1479), is a potent inhibitor of the *in vitro* replication of the hepatitis C virus (HCV) [25]. Once the parent compound has been delivered in the host cell and has been phosphorylated to its 5'-triphosphate metabolite, it acts as an inhibitor of the viral RdRp (Figure 1). The efficacy of balapiravir was demonstrated in patients chronically infected with HCV [26,27]. The clinical development of this compound for the treatment of chronic HCV was halted because of an unacceptable benefit to risk ratio [28]. Since the RdRp of DENV shows similarities with that of HCV, it was anticipated that the drug would also be effective against DENV. Balapiravir was indeed shown to inhibit the replication of various DENV serotypes and strains (both lab and clinical) with EC₅₀ values ranging from 1.9 to 11 µM in human hepatoma (Huh-7) cells and 1.3 to 6.0 µM in primary human macrophages and dendritic cells, as determined by RT-qPCR [29]. The antiviral activity was confirmed in peripheral blood mononuclear cells (PBMCs) with EC₅₀ values ranging from 0.10 to 0.25 µM [30]. On the basis of these observed activities, a DENV therapeutic trial was designed in which the drug was dosed orally at 1500 and 3000 mg BID. This led to median plasma exposures of 3.56 µM and 5.85 µM, respectively, which is 1.6-fold and 2.6-fold higher than the EC₅₀ value obtained in macrophages [29]. Although balapiravir proved to be safe at the doses administered, it was unable to cause significant changes in viral load and viremia kinetics, cytokine expression levels, nor in hematological and/or biochemical parameters in dengue-infected patients [29]. Several reasons have been suggested for the lack of potency. Firstly, the

compound may be less efficacious at a time point at which viremia has reached peak levels. Indeed, when added after the onset of *in vitro* infection, balapiravir was 52-fold less effective in PBMCs [30]. Not only was the compound less active when added after infection, the activity of balapiravir also appears to be cell type-specific and is significantly less effective in Huh-7 and adenocarcinomic human alveolar basal epithelial (A549) cells and to a lesser extent in human monocytic THP-1 cells than in primary cells [30]. This might be due to variations in the phosphorylation potential (converting balapiravir to its active triphosphate metabolite) of the different cells. Similarly, the potential to convert balapiravir into its 5'-triphosphate form was also shown to be affected by cytokines that were released upon DENV infection, leading to a significant decrease in the *in vitro* antiviral potential when the compound was added after infection [30]. As a result, the obtained EC₅₀ values were higher than the median plasma exposures, which might be one of the main reasons that balapiravir failed in the clinical trial. Higher plasma exposures could be achieved either by increasing the dose or the dosing frequency, which may however result in the appearance of adverse effects. Indeed, at a dose of 4500 mg twice daily for 2 weeks, balapiravir caused modest to severe side effects in HCV-infected patients, including headache, dizziness, vomiting, nausea, diarrhea, abdominal pain, back pain, and neck pain [26]. Increasing the dose or dosing frequency is therefore not an option to improve the antiviral efficacy of balapiravir in DENV-infected patients. Moreover, the fact that the compound also proved inactive in a mouse model of DENV infection, even at a dose of 200 mg/kg/d [30], indicates that the compound may not be suitable to treat DENV-infected patients.

Chloroquine

As a (rather poor) base, chloroquine (PubChem CID: 2719) is capable of alkalinizing the acidic environment of intracellular organelles, such as the endosome, lysosome and vesicles of the Golgi complex [31]. This quality renders it suitable to hinder the entry of (dengue) viruses that, for release into the cytosol, rely largely on the acidic pH-mediated fusion of the viral membrane with that of the endosome, and/or to impair furin-dependent virus maturation in the low-pH setting of the Golgi

(Figure 1) [32-34]. In addition, chloroquine was shown to possess anti-inflammatory properties by lowering both the secretion of TNF- α and IL-6 as well as inhibiting the expression of the TNF- α receptor [35]. This suggests that the drug might potentially be efficacious against viruses that are implicated in triggering pro-inflammatory pathways or even a cytokine storm as is the case in the pathogenesis of dengue. In African green monkey kidney (Vero) and human macrophage (U937) cells, the compound was shown to inhibit DENV-2 replication in a dose-dependent manner at non-toxic concentrations. No antiviral activity was, however, noted in infected *A. albopictus* C6/36 cells [36,37]. In DENV-infected U937 cells chloroquine reduced the expression levels of pro-inflammatory cytokines (IFN- α , IFN- β , IFN- γ , TNF- α , IL-6 and IL-12 [37]). Chloroquine-mediated inhibition of IFN- α expression was also observed in DENV-infected plasmacytoid dendritic cells [38], suggesting that the compound may modulate the cytokine response against this virus. Although chloroquine appears to bring about beneficial effects against DENV infection *in vitro*, it seems to be less equipped to combat DENV infections in patients [39,40]. A 3-day chloroquine treatment had no favourable effect on viremia, NS1 antigenemia or fever duration, nor on the cytokine or T cell response [39]. However, a trend towards a smaller, though non-significant, number of patients developing dengue hemorrhagic fever was observed for patients treated with chloroquine compared to those in the placebo group. A second, small clinical study conducted in Brazil confirmed that chloroquine is not potent enough in dengue patients; no significant impact on the duration of dengue disease or fever was observed [41]. On the other hand, patients treated with chloroquine did report on experiencing less pain and were better capable of performing all-day activities, hence, it may improve the quality of life [41].

Lovastatin

Statins inhibit numerous cellular pathways, including cholesterol synthesis. Cholesterol is involved in the replication cycle of a variety of viruses, including those belonging to the *Flaviviridae* [42-46]. Lovastatin (PubChem CID: 53232) was reported to inhibit *in vitro* DENV replication in various cell types and was shown to exert its activity at the late stages of the DENV replication cycle (maturation and

egress), thereby reducing the release of infectious viral progeny (Figure 1) [47-49]. Surprisingly, the compound was also shown to block viral entry (Figure 1) [48,50]. The fact that this compound inhibits different mechanisms points towards a pleiotropic/aspecific antiviral effect. In DENV-infected AG129 mice, lovastatin treatment resulted in a very modest delay (~ 2 days) in virus-induced mortality, which was irrespective of the time point at which treatment was initiated, that is pre-infection or post-infection [51]. This minor effect on mortality was not reflected in a reduction of viral RNA levels (the maximum reduction observed was 0.1 log₁₀; in some mice even an increase in viral titers was observed). The fact that this widely used drug with an excellent safety record is also endowed with a beneficial effect on the endothelium (i.e., restoring endothelial function, promoting endothelial cell migration and proliferation, and inhibiting inflammatory pathways) [52-55] motivated to initiate clinical studies to assess its effect on DENV infections [56]. The study was performed in adult, laboratory-confirmed dengue patients within 3 days that they presented with fever [57]. Overall, lovastatin treatment (80 mg lovastatin, QD, 5 days) did not lower viral RNA levels, nor did it accelerate virus clearance or offered symptomatic relief [57]. There was some tendency towards a slightly lower viremia in patients infected with DENV-2. Also, in patients experiencing a secondary infection, the virus was cleared slightly faster in the lovastatin treatment group. Both trends, however, were found to be non-significant [57].

Prednisolone

Since pro-inflammatory responses play a pivotal role in dengue pathogenesis, corticosteroids may alleviate symptoms that are associated with severe dengue (capillary permeability, hemorrhagic shock, thrombocytopenia and hemorrhage). Hence, corticosteroids may be administered in addition to antiviral therapy to inhibit or even prevent disease progression. Because the (short term) use of corticosteroids at a low dose (≤ 2 mg/kg/d) is devoid of any serious side effects, combined with the observation that corticoids do not enhance *in vitro* dengue virus replication, a clinical trial was initiated to study whether short-term oral treatment (0.5 or 2 mg/kg/d, for 3 days) with prednisolone is safe to

use during the early acute phase of dengue infection [58]. Although safe to use, corticosteroid therapy was not efficacious in pediatric dengue patients who had fever for less than 3 days. Prednisolone did not significantly lower viremia or increase virus clearance, nor did it have an advantageous effect on fever clearance, alanine aminotransferase (ALT) and plasma albumin levels, and coagulation [58]. Possible reasons for the failure may have been that treatment was started at a time point that dengue infection was already well established and/or the administered doses were too low [58,59].

Celgosivir

Celgosivir or 6-*O*-butanoyl castanospermine (PubChem CID: 60734) exerts *in vitro* antiviral activity against a number of viruses, including DENV ([60-63], Kaita K *et al.*, abstract in J Hepatol 2007, 46:S56). It inhibits the *in vitro* replication of DENV1-4 clinical isolates with EC₅₀ values in the sub- μ M range (0.22 – 0.68 μ M); concentrations that are well below the toxic concentration of the compound. The anti-DENV activity appears to result from two mechanisms. Firstly, the compound acts as an inhibitor of endoplasmic reticulum (ER) α -glucosidases, thereby impairing proper processing of the DENV E, prM and NS1 proteins (Figure 1) [62,63]. Secondly, the drug modestly modulates the host unfolded protein response (UPR) pathway, thereby changing expression levels of pro-survival as well as pro-apoptotic proteins [62]. Oral treatment of DENV-infected AG129 mice with celgosivir (75 mg/kg, BID, for 3 days) resulted in a viral RNA load reduction of 88% at the peak of viremia (on day 3 pi) [64]. *In vivo* efficacy was confirmed in a lethal challenge mouse model of DENV infection, showing that celgosivir treatment (at a dose of 50 mg/kg, BID, for 5 days) caused a significant reduction in viremia at day 1 and day 3 pi and resulted in full protection against virus-induced mortality in infected mice [63]. However, in a phase 1b clinical trial (CELADEN) celgosivir failed to show a significant antiviral effect, although modest antiviral trends were observed. In patients with secondary DENV infection the mean viral RNA load in the serum as well as serum levels of NS1 were lower in the celgosivir-treated group than in the placebo group [65•]. Intriguingly, the authors noticed a therapeutic difference between patients undergoing either a primary or a secondary infection. Since this difference was unexpected, the study might have

been underpowered to detect a potential significant therapeutic effect of celgosivir in patients with a secondary dengue virus infection [65•]. Additional reasons for the protective failure might be the fact that the antiviral activity of celgosivir turned out to be both virus strain-dependent and cell type-dependent with EC₅₀ values in the high μ M range for DENV-1 strain 2402 (EDEN1) in Huh-7 and Vero cells, in particular [66]. Virus-strain dependency of the antiviral efficacy of celgosivir was also observed in mice. Although celgosivir (50 mg/kg, BID, oral administration) significantly reduced the viral RNA load in AG129 mice infected with either DENV1-2402 (EDEN1), DENV2-3295 (EDEN2) or DENV-2 S221 on day 3 pi, the decrease in viremia was ~4-fold and ~7-fold less in mice infected with EDEN1 and S221, respectively [66]. In mouse studies, in which the compound was shown to be effective, treatment was started at the day of infection, which may somehow mimic a situation of pre-exposure prophylaxis (PrEP). When treatment was first initiated at the time of peak viremia in mice, much higher doses/frequencies of administration was required to result in a reduction of viremia [66]. In the clinical studies, the compound was given to patients with an ongoing infection. In this context, celgosivir may possibly not be sufficiently potent, explaining to some extent the relative lack of activity in the therapeutic trial. Possibly, with an optimized dosing schedule, a more pronounced effect of the compound in DENV-infected patients may be observed.

Conclusions

In the recent decade, an absolute revolution has been achieved in the development of highly efficacious and safe antiviral treatments for HCV. In fact today, combinations of two or more highly potent directly acting HCV antivirals result in a high sustained virological response/cure in >95% of the patients following only 5 to 8 weeks of oral therapy [67,68•,69-73]. Since HCV and DENV belong to the same family of viruses, we believe that it should be, provided sufficient efforts, very well feasible to also develop highly potent, selective and safe DENV inhibitors that should have pan-serotype activity. So far, however, very few directly acting antivirals have been reported against DENV or any other flavivirus. Given the enormous impact that dengue has on public health in endemic regions and the

complexity associated with DENV vaccine development, there is a real need for small molecule DENV drugs that can be used for the prophylaxis and treatment of infections of this virus. In the absence of specific inhibitors, the potential effect of (i) 3 host-targeting inhibitors of DENV replication (chloroquine, lovastatin and celgosivir) as well as (ii) that of prednisolone and (iii) the HCV polymerase inhibitor balapiravir has been assessed in DENV-infected patients. No obvious beneficial clinical effect was noted with any of these drugs, emphasizing once again the urgent need for highly potent directly-acting antivirals (DAAs). The lessons learned when carrying out these therapeutic trials may help to guide the design of trials for the next generation of DENV inhibitors, that is DAAs. Simmons and colleagues [74•] formulated recommendations for designing and performing early clinical trials. The question then remains whether assessing the potential efficacy of novel drugs in phase IIa studies should best be carried out in naturally infected patients or in human DENV challenge models [75-79], and whether or not to explore the potential efficacy first in a prophylactic or therapeutic setting. In conclusion, today, there is neither a vaccine for the prevention, nor a drug for the treatment or prophylaxis of a disease that sickens close to 100 million people annually. Although dengue is an acute infection, unlike hepatitis C, leaving only a limited time window for therapeutic intervention, the great successes achieved in the battle against HCV should inspire and give hope that potent dengue antivirals may be successfully developed.

Conflict of interest statement

Nothing declared.

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Legend to the Figure

Figure 1. Schematic representation of the DENV life cycle. Compounds that were evaluated in dengue clinical trials target different steps of the DENV replication cycle. Both lovastatin and chloroquine target early as well as late steps of viral replication: lovastatin inhibits virus entry and virus maturation and egress. Chloroquine inhibits the fusion of the viral membrane with that of the endosome as well as virus maturation; both processes are largely driven by the acidity within the cellular compartments. As an α -glucosidase inhibitor, celgosivir impairs proper glycosylation of the DENV E, prM and NS1 proteins. The nucleoside analog balapiravir directly targets viral RNA synthesis by acting as a chain terminator.

Figure 1