Drug repurposing approaches to fight Dengue virus infection and related diseases

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1. ABSTRACT

Dengue is a mosquito-borne viral disease caused by four antigenically distinct serotypes of Dengue Virus (DENV), namely DENV1–4 and is currently considered the most important arthropod-born viral disease in the world. An effective antiviral therapy to treat Dengue Virus infection is still missing and a number of replicative cycle inhibitors are currently under study. Considering the rapid spreading of DENV and the common timeframe required for bringing a new drug on the market, the repurposing of approved drugs used for different diseases to identify novel inhibitors of this pathogen represents an attractive approach for a rapid therapeutic intervention. Herein, we will describe the most recent drug repurposing approaches to fight DENV infection and their implications in antiviral drug-discovery.

2. INTRODUCTION

The Flaviviridae family of viruses consists of more than 70 different hematophagous arthropod-borne human pathogens. The best known pathogens of this family are West Nile Virus (WNV), Yellow Fever Virus (YFV), Japanese Encephalitis Virus (JEV) and the four serotypes of Dengue Virus (DENV 1–4) (1). The mosquito-vectored Dengue virus represents a leading cause of illness and death in the tropics and subtropics, and in recent years, it has expanded in other geographical areas including Asia, Africa, and the Americas. In this context, the World Health Organization (WHO) has drawn a dramatically different scenario from what prevailed 20 or 30 years ago, indicating that nearly half of the human population lives in risk areas, with 390 million infections and around 20.000 deaths each year (2). All the known vectors of DENV are mosquitoes belonging to Aedes genus, especially A. aegypti, A. albopictus, and A. polyniensis, and become infected when they feed on humans during the usual five-day period of viraemia. After 3–6 days of incubation, the patient experiences symptoms like lethargy, headache, myalgia, nausea, vomiting and rash, before complete recovery. Sometimes, the disease can progress to potentially fatal Dengue Hemorrhagic Fever (DHF), characterized by increased vascular permeability, plasma leakage and bleeding, which lead to Dengue Shock Syndrome (DSS) (3). The incidence of severe disease is most likely to happen during secondary infections, usually due to other serotypes of the virus, meaning that immunity needs to be induced against all four existing DENV serotypes over a prolonged period.

Dengue is fast emerging as a pandemic-prone viral disease and infections in international travelers returning from highly endemic areas are increasing. In temperate European countries, where the Dengue virus is not endemic, international travelers, climate changes and the presence of Dengue vectors could modify the present situation. Thus, domestic outbreaks of Dengue fever originating from imported cases have to be considered as a possible risk. The expanding geographical distribution of both the virus and the mosquito vector, the increased frequency of epidemics, and the emergence of Dengue Hemorrhagic Fever in new areas, prompted the WHO to classify Dengue as a major international public health concern.

Currently, despite global efforts, there is no clinically approved antiviral therapy against DENV infections and only symptomatic treatment and supportive care in a hospital setting are available for patients (4). It follows that the development and application of targeted strategies to fight Dengue virus infection are extremely important and urgent. On the other hand, a Dengue vaccine is available in Mexico only for patients aged 9–45 living in endemic areas, but is not been foreseen for children <9 years or travelers (5). The existence of multiple DENV serotypes and the underlying mechanism that leads to DHF and DSS could severely limit the overall efficacy of the vaccine. Moreover, the price of DENV vaccines for the public sector would be a major determinant of whether or not governments would introduce the vaccine. Therefore, a small-molecule delivered early or even taken prophylactically, is expected to have a significant impact on the prevention and progression of the disease by lowering the viral load in the blood, the number of patients that will progress to DHF/DSS, and lastly, the number of carrier mosquitoes that become infected after feeding on a viraemic patient, thereby slowing down transmission.

As discussed in the next chapters, several approaches are currently under study for the discovery of small-molecule drugs able to block DENV replication, which are mostly focused on targeting viral/host proteins and on the repurposing of known drugs.

3. DENV REPLICATION AND BIOLOGICAL TARGETS

DENV is a single-stranded RNA virus that is approximately 11 kb in length and is released into the cytoplasm immediately after cell entry. The viral genome encodes for three structural proteins, capsid (C), pre-membrane (prM) and envelope (E) and for seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). Host cell invasion by DENV starts with the interaction of the E protein with a superficial cellular receptor still unknown. After the first interaction, the virion is transported into the cytoplasm by a chlatrin-mediated endocytosis (6). After rearrangement of E protein, required for fusion of viral and cellular membranes, the RNA genome is released
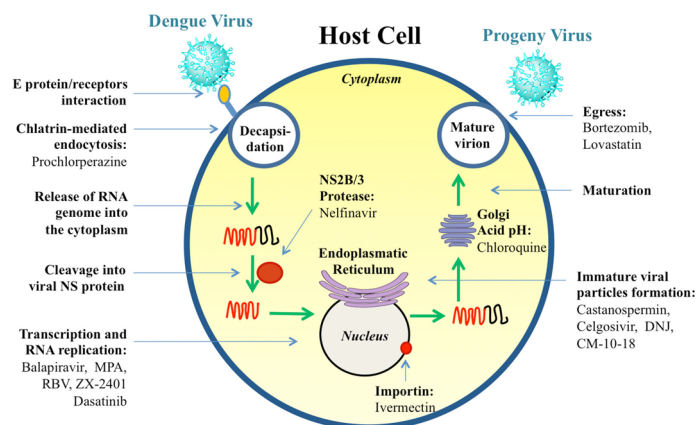
**Figure 1.** Dengue virus life cycle and steps inhibited by repurposed drugs.

into the cytoplasm (7). This rearrangement is due to a change in pH generated by the endocytic vesicle containing the virion (8). Once released, genomic RNA is translated into a long polyprotein, which is then cleaved by NS2B/3 viral protease into viral NS proteins. Transcription starts when these proteins are translocated into the endoplasmatic reticulum (ER) close to the replication site (9). The newly formed RNA associates with C, prM and E proteins during the budding into the lumen of the ER and forms immature viral particles (10). These particles enter the secretory pathway, pass from the low pH of the trans-Golgi to the neutral pH of the extracellular milieu, lose the soluble pr peptide from the prM protein and, once outside the cell, are able to infect another cell (11). Molecular understanding of the viral life cycle and the viral-host interaction has enabled discovery of small-molecule and peptide inhibitors targeting virus-cell fusion, RNA processing, genomic replication, virion assembly and final budding of the mature virus (Figure 1) (4,12–14).

Different classes of drug-candidates have been developed in the last decade, which target *i)* viral entry, like NITD-488 that inhibits the fusion of the viral and the cellular membranes mediated by E protein (15); *ii)* viral NS2B-NS3 protease, like Cpd1 identified by High-Throughput Screening (HTS) (15b); *iii)* helicase C-terminal domain of the NS3 protein, like ST-610 (15c); *iv)* RNA methyltransferase (MTase) and RNA-dependent RNA polymerase (RdRP) activities of the NS5 protein, like aurintricarboxylic acid (ATA) (15d); *v)* NS4B protein, that is vital for the replication of viral genome, like lycorine (16); *vi)* host cell kinases (Src/Fyn) required for RNA virus replication, like the anticancer drug Dasatinib (Figure 2) (17).

4. DRUG REPURPOSING FOR DENGUE

Drug repurposing, drug repositioning or drug re-profiling is the identification of new therapeutic indications for already known drugs. These drugs can be approved or marketed compounds used in a different clinical setting, or they can be derivatives that did not succeed in any stages of the clinical trials for different reasons. In one sentence, drug repurposing is the discovery of new indications for approved or failed drugs (18). The importance of the drug repurposing approach is represented by a faster and safer way to develop medications against new diseases or diseases for which a therapeutic treatment is still unavailable. Pharmaceutical companies are also motivated to invest in such drug repurposing programs, since the risk of clinical failure is lower and the investments needed to reach the market are very much reduced. In fact, it is known that *de novo* drug discovery and development is a 10–17 years process from the idea to the marketed drug (19–20), and that the probability to succeed is lower than 10% (21). Drug repurposing offers the possibility to reduce time and risks intrinsic to any drug discovery process and to quickly advance a drug-candidate to late-stage development. With this strategy, in fact, several phases of the de novo drug discovery and development can be avoided being already documented in the original indication of the re-profiled candidate. It further offers the possibility to expand the market and to extend the application or patent life of a drug. Drug repositioning can be considered a first line approach also for rare and neglected diseases or diseases primarily occurring in developing countries, where an effective treatment is urgently needed, especially because these conditions are difficult to address for financial reasons. Finally, drug repurposing offers the possibility to develop multitarget drugs able to interfere, at the same time, with two or more pathways/targets involved in the pathogenesis of the same disease or in the progression of highly-related diseases (e.g. co-infections). Multitarget drugs may therefore have multiple advantages: *i)* a synergistic effect, allowing to use lower doses and thus reducing

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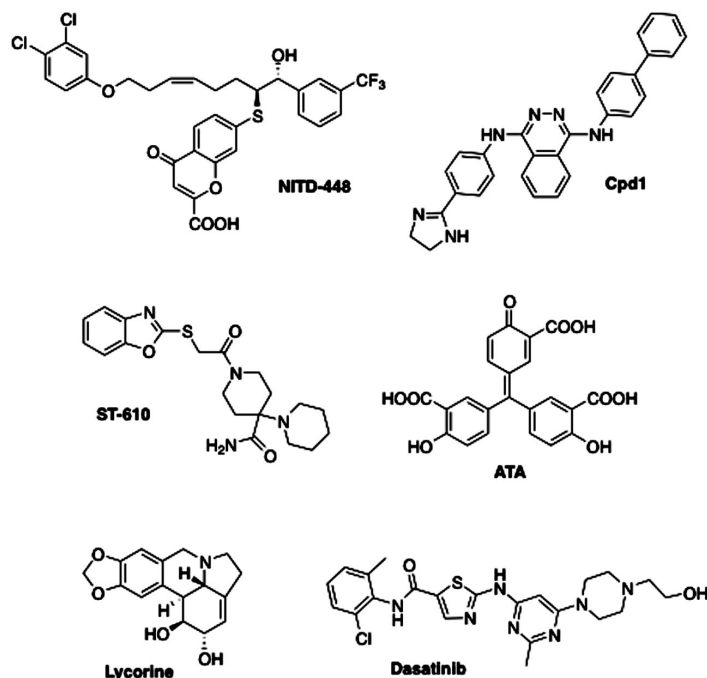
Figure 2. Molecular structure of representative DENV inhibitors targeting viral and host proteins.

the risk of side effects; *ii)* a reduced drug-resistance, acting on different targets (e.g. a viral and a host cell factors); *iii)* a better compliance, being administered as single-pill agents; *iv)* a simplified pharmacokinetic and pharmacodynamic profile and a reduced risk of drug−drug interactions (22–23). As an example, our research group has recently developed a first-in-class family of multitarget drug-candidates able to inhibit DENV replication by acting on host kinases (Src/Fyn) and viral proteins (NS5-NS3 interaction) (23b). In the next paragraphs, a series of representative examples will be reported to describe the repositioning of known drugs for the inhibition of DENV replication (Table 1).

4.1. Antiviral drugs

4.1.1. Nelfinavir

Nelfinavir (AG1343) is a potent protease inhibitor active against human immunodeficiency virus 1 (HIV-1) (24). It is orally bioavailable and it is usually used in combination with other antiretroviral drugs. Nelfinavir showed also anti-hepatitis C virus (HCV) (25) and anticancer (26) activity. Nelfinavir and other viral protease inhibitors like Lopinavir and Ritonavir were chosen for a computer aided drug design and molecular modeling campaign aimed at repositioning peptidomimetics against Dengue virus infection (27). Using molecular docking calculations and molecular dynamics simulations, favorable interactions between Nelfinavir and DENV NS2B-NS3 protease were identified. Next, a virus-cell-based assay conducted

on Vero-B cells infected with DENV serotype 2 strain New Guinea C confirmed that Nelfinavir possesses an acceptable antiviral activity against DENV (EC_{50} = 3.5 ± 0.4 µM and a selectivity index (SI) of 4.6). Even if the potency of this derivative is moderate, the structural and pharmacophoric features delineated in this study could be a good starting point for the development of novel and more potent Dengue protease inhibitors.

4.1.2. Balapiravir

Balapiravir is a tri-isobutyrate ester prodrug of 4'-azidocytidine (R1479), a nucleoside analogue able to inhibit HCV RNA-dependent RNA polymerase. The corresponding 5'-triphosphate derivative of R1479 is a potent inhibitor of HCV replicase blocking the RNA synthesis as a cytidine triphosphate-competitive inhibitor with a K_i of 40 nM (28). In addition, this nucleoside analogue is efficacious against Huh-7 DENV infected cells with an EC_{50} of 1.9–11.0 µM, primary human macrophages (EC_{50} = 1.3–3.2 µM) and dendritic cells (EC_{50} = 5.2–6.0 µM) (29). Starting from *in vitro* data available for R1479, the precursor Balapiravir was used in a double blind randomized placebo-controlled trial conducted in adult male patients infected by DENV and having fever for less than 48 hours (30). Two groups of patients receiving placebo and two groups receiving a dose of 1500 mg and 3000 mg of Balapiravir twice a day for 5 days were involved in this study. Both, control and Balapiravir receiving patients suffered the same mild adverse effects, meaning that the drug is well tolerated. For patients

Table 1. Drugs repurposed as DENV inhibitors

Chapter	Drug Class	Drug	Original Activity	DENV activity
4.1.	**Antivirals**	Nelfinavir	Protease Inhibitor	NS2B-NS3 protease inhibition
		Balapiravir	Nucleoside Analogue	RNA-dependent RNA polymerase inhibition
		Mycophenolic and Ribavirin	Inosine Monophosphate Dehydrogenase (IMPDH) inhibitor	Guanosine depletion
		ZX-2401	Nucleoside Analogue	Guanosine depletion
4.2.	**Antimalarics**	Chloroquine	Increases the pH of acidic organelles	Inhibits low-pH dependent entry steps
		Amodiaquine	Heme-polymerase inhibition	
4.3.	**Antidiabetics**	Castanospermin	ER-glucosidase I inhibition	prM and E folding interference
		Celgosivir	ER-glucosidase I inhibition	Accumulation of NS1 in ER
		Deoxynojirimycin	ER-glucosidase I inhibition	Inhibition of virus budding from ER
		CM-10–18	Glucosidase I and II inhibition	Viral glycoproteins folding interference
4.4.	**Antihistamines**	Cromolyn, Montelukast, Ketotifene	Mast cells modulators	Reduction of vascular leakage DENV-induced
4.5.	**Anticancers**	Dasatinib and AZD0530	Kinases inhibitors	Src Fyn kinases inhibition
		Bortezomib	Proteasome inhibitor	DENV egress inhibition
4.6.	**Antipsychotics**	Prochlorperazine	D2 receptor antagonist	Clathrin-mediated inhibition
4.7.	**Antiparasites**	Ivermectin	Permeability and hyperpolarization increase	NS3 helicase activity inhibition
		Suramin	Parasite metabolism inhibition	NS3 helicase inhibition
		Nitazoxanide A	Pyruvate-ferredoxin oxidoreductase interference	Mechanism not well understood
4.8.	**Anticholesterols**	Lovastatin	HMG-CoA Reductase inhibitor	Membrane Cholesterol reduction
4.9.	**Steroids**	Dexamethasone	Anti-inflammatory and immunosuppressant agent	Platelet count increase
		Prednisolone	Synthetic glucocorticoid	Antiinflammatory and antihemorrhagic activity
4.1.0	**Antibiotics**	Geneticin	80S ribosome interference	Inhibition of E formation
		Narasin	Ionophore	E and NS5 expression reduction
		Minocycline	Anti-inflammatory and immunomodulatory effects	ERK1/2 inhibition and IFN-α upregulation
4.1.1	**Antiarrhytmics**	Lanatoside C	Na^+-K^+-ATPase pump inhibition	Mechanism not well understood

treated with 3000 mg Balapiravir, the plasma drug concentration during the first 12 hours is comparable to the effective *in vitro* concentration of Balapiravir. However, virological markers, fever clearance time, plasma cytokine concentrations and the whole blood transcriptional profile were not attenuated by the treatment. The mild or null efficacy of Balapiravir *in vivo* was studied later in more detail in order to explain the differences observed between the *in vitro* and *in vivo* activities (31). Early treatment with R1479 showed a potent inhibitory activity on DENV polymerase (EC_{50} = 0.103 µM in infected Peripheral Blood Mononuclear Cells; PBMC), but delayed treatment decreased the potency by 125-fold (EC_{50} = 12.85 µM), when R1479 was added 24h post infection. This result depends on the negative influence of cytokines on the conversion of R1479 to its triphosphate. In fact, pretreatment of PBMC with IP-10, TNF-α, IL-10, or GM-CSF decreased

the potency of R1479 by 6.3- to 19.2-fold. Also the use of higher doses of Balapiravir did not produce the desired effect. Even when it was dosed in a mouse model at up to 100 mg/kg twice daily for 3 days, a marked reduction in viremia was not registered. These results indicated the reasons why Balapiravir failed the clinical trial and why this nucleoside analogue is not successful against Dengue infection.

### 4.1.3. Mycophenolic acid and ribavirin

Mycophenolic Acid (MPA) is a potent and non-competitive inhibitor of Inosine Monophosphate Dehydrogenase (IMPDH), a key enzyme required for the biosynthesis of guanine nucleotides (32). The immunosuppressive activity of MPA is mainly used to prevent organ rejection in transplantation, in fact, it probably acts by blocking T cell proliferation. Previous
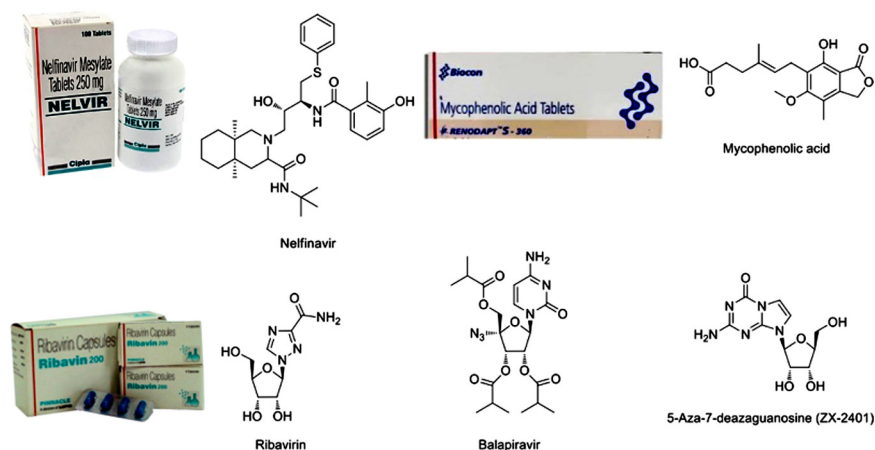
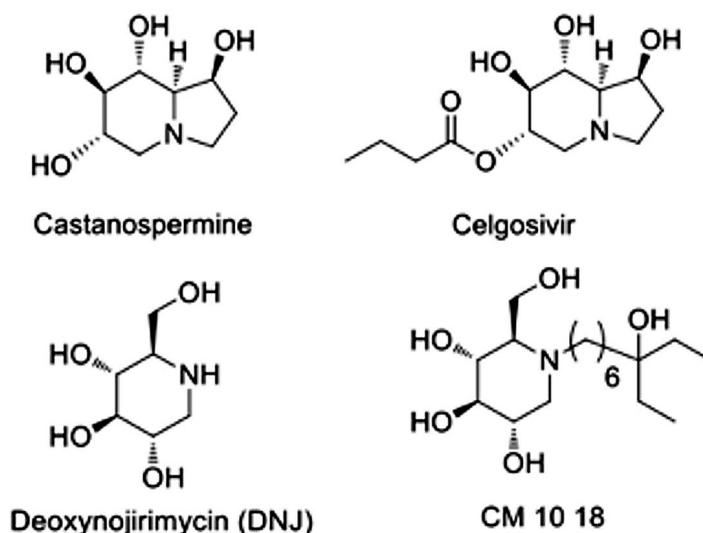**Figure 3.** Antiviral drugs repurposed as DENV inhibitors
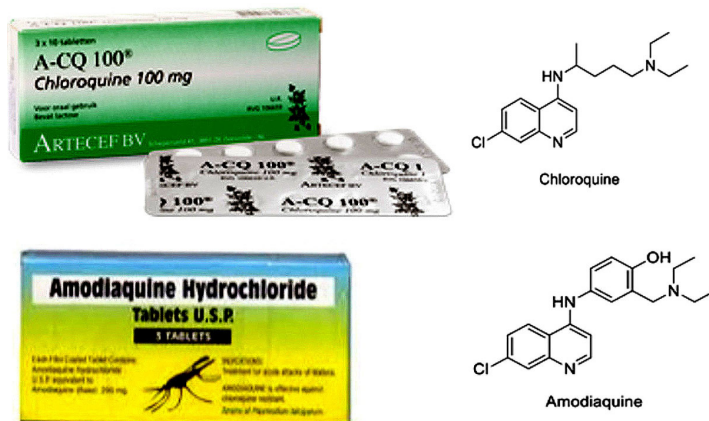
Figure 4. Antimalaric drugs repurposed as DENV inhibitors

studies demonstrated that MPA is able to inhibit *in vitro* infections with several viruses by blocking the synthesis of xanthosine monophosphate and consequently depleting the intracellular guanosine pool.

Ribavirin (RBV) is a guanosine analogue with a broad-spectrum antiviral activity mostly used against Human Respiratory Syncytial virus (RSV) and HCV. It is phosphorylated by cellular kinases at the 5′-position after entering into the cell, like it happens with the other nucleoside analogues. Ribavirin 5′-monophosphate inhibits the IMPDH, reducing the intracellular quantity of guanosine nucleotide, which adversely affects the processivity of the viral replicase. Moreover, the antiviral effect of Ribavirin nucleotides may be due to mutagenesis of the viral genomes (33).

These two antiviral agents were tested against Dengue virus in two different studies (32, 34). Both studies showed that MPA is active at a concentration lower than RBV: treatment of hepatic cells expressing DENV-2 with MPA showed an approximately 65-fold greater potency than RBV, with an IC_{50} of 0.1 µg/ml (0.31 µM). At the clinically therapeutic drug level, MPA (10 µg/ml, 30 µM) reduced virus production by greater than 6 log, whereas RBV (25 µg/ml, 100 µM) decreased it by 3 log. The inhibition was not due to a toxic effect, since there was no difference in cell viability by trypan blue exclusion after treatment with MPA at the doses used. At high concentrations, both MPA (10 µg/ml) and RBV (50 µg/ml) had a mild cytostatic effect, as they inhibited cell growth by 50%. In agreement with previous studies the inhibitory effects were completely reversed by the addition of exogenous guanosine. To confirm these effects, three other hepatoma cell lines (Huh-7, CRL-8024, and HepG2) were exposed to DENV-2, treated with MPA and RBV, and analysed: also in this case, MPA showed a higher potency compared to RBV (32). In addition, time-course analyses on Hep3B infected

Figure 5. Antidiabetic drugs repurposed as DENV inhibitors
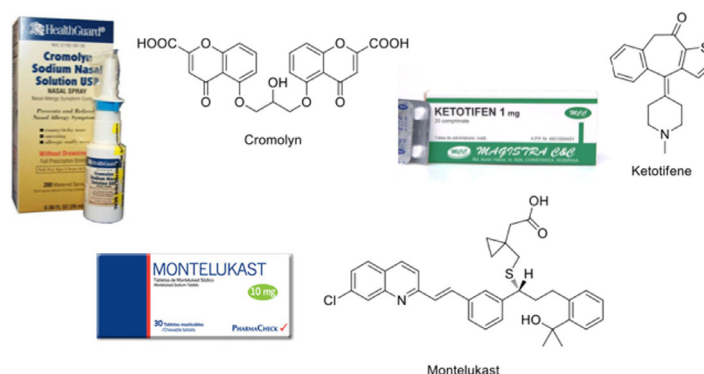
**Figure 6.** Antihistamine drugs repurposed as DENV inhibitors

cells and quantitative real-time RT-PCR established that DENV infection could be prevented *in vitro* by pharmacologically relevant concentrations of MPA that do not block the translation of the input strand of the viral RNA but prevent the synthesis of viral RNA (32). A second analysis found comparable results, showing that RBV and MPA inhibit DENV-2 replication in monkey kidney cells (LLC-MK2) with an IC_{50} = 50.9±18 µM and IC_{50} = 0.4±0.3 µM respectively (35). After treatment with both, RBV (200 µM) and MPA (10 µM), the production of defective viral RNA consistently increased, as revealed by quantitative real-time RT-PCR of viral RNA and plaque assays of virions from DENV-2 infected cells. In addition, a marked decrease of the viral replicase activity was registered. RBV may also compete with guanine-nucleotide precursors in viral RNA translation, replication and 5' capping (35). All these results demonstrated that these two agents are able to inhibit DENV infection by preventing synthesis and accumulation of viral RNA.

4.1.4. ZX-2401

ZX-2401, a nucleoside analogue containing a bridgehead nitrogen atom in the purine ring, was originally synthesized and tested against Picornaviruses (36). The mechanism of action of ZX-2401 is currently unknown, but due to the structural analogy with natural nucleosides, the mechanism should be similar to RBV.

The initial biological evaluation highlighted a pronounced activity of this compound against Vesicular Stomatitis Virus, Echo-6 Virus and Coxsackie B-1 Virus, and a moderate, but comparable to RBV, activity against five rhinoviruses (36). These data encouraged a successive evaluation of ZX-2401 against other RNA-viruses, like those belonging to the Flaviviridae family. In a second round of studies, ZX-2401 showed an activity equivalent to RBV against Yellow Fever Virus (YFV) and Bovine Viral Diarrhea Virus (BVDV),
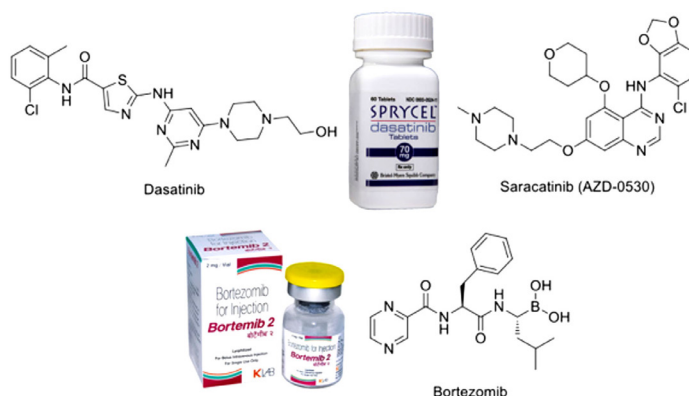
Figure 7. Anticancer drugs repurposed as DENV inhibitors

Figure 8. Antipsychotic agents repurposed as DENV inhibitors

but superior to RBV against Banzi virus (BV), Dengue Virus (DENV) and West Nile Virus (WNV). In particular, ZX-2401 showed remarkable activity in a Cytopathic Effect Assay (CPE) against DENV replication *in vitro*. In this experiment, the EC_{50} value registered for ZX-2401 was 10 µg/ml compared to >80µg/ml of RBV. Moreover, ZX-2401 completely inhibited DENV replication in cell culture at a concentration of 32 µg/ml and with low toxicity (37).

4.2. Antimalaric drugs

4.2.1. Chloroquine

Chloroquine is an antimalarial agent that has also been used for treatment of rheumatoid arthritis, systemic lupus erythematosus and in systemic therapy of amebic liver abscesses (38). It is a 9-aminoquinoline, acting as a diprotic weak base able to increase the pH of acidic organelles such as endosomes, Golgi vesicles and lysosomes (38). Chloroquine inhibits low-pH dependent entry steps or interferes with post-translational modifications of newly synthesized proteins important for flavivirus replication (e.g. the transmembranal envelope protein M), especially blocking the glycosylation (39). Chloroquine was involved in many drug repositioning studies in the DENV scenario. It was proved to be able to inhibit Dengue

Virus Type 2 replication in Vero cells at a dose of 5 µg/mL (500 µg/mL is the cytotoxic dose) as revealed by plaque assay and qRT-PCR (40). In a second study it was found that at a higher dose (50 µg/mL), this antimalarial agent was not toxic to infected U937 cells, but able to reduce virus production in comparison to untreated cells. It also caused a significant reduction in the expression of proinflammatory cytokines like IFN-ß, IFN-γ, TNF-α, IL-6 and IL-12 (41). The grade of inhibition of replication of DENV-2 was evaluated also *in vivo* and specifically in Aotus Monkeys (42). The sera of treated animals after infection were analysed, in order to register the biochemical and hematological parameters, like alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (AP). Flow cytometry and Cytometric Bead Array were used for quantification of cytokines IFN-γ, TNF-α, IL-2, IL-6, IL-4, IL-5. Analyses of the sera highlighted that Chloroquine is effective in inhibiting DENV-2 replication and induces a reduction of viremia compared to controls. A marked reduction in the amount of IFN-γ and TFN-α was observed following the treatment with Chloroquine after infection. Moreover, decrease in systemic levels of liver enzyme AST was monitored (43). Finally, Chloroquine was also used in a vast randomized controlled trial, on 307 adult patients, for treatment of DENV (44). From this study, clear evidence emerged that this antimalaric agent could
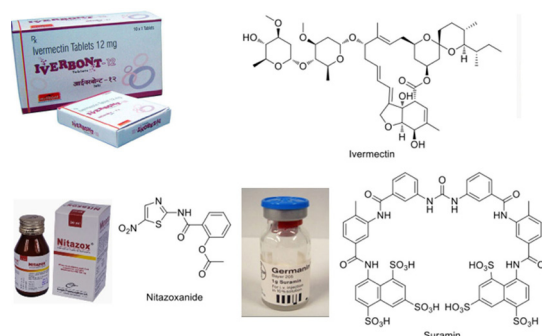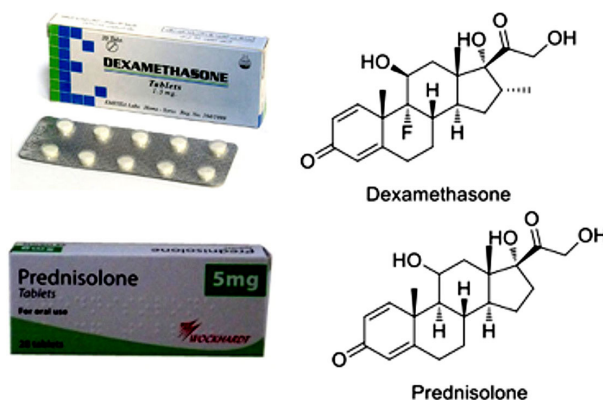
Figure 9. Antiparasite drugs repurposed as DENV inhibitors

Figure 10. Anticholesteremic agents repurposed as DENV inhibitors

Figure 11. Steroid drugs repurposed as DENV inhibitors

reduce the duration of viraemia or NS1 antigenaemia in adult Dengue patients. An anti-pyretic activity was also observed. Unfortunately, Chloroquine generated more adverse events than placebo, even if they were usually of mild entity. In addition, Chloroquine did not attenuate cytokines or T cell responses to DENV infection (44).

4.2.2. Amodiaquine

Amodiaquine is an orally active antimalarial and anti-inflammatory agent, structurally similar to Chloroquine. Its mechanism of action has not been fully elucidated yet, but it seems to inhibit the heme-polymerase activity generating accumulation of free heme, which is toxic for the parasites (45). Amodiaquine decreased DENV-2 infectivity with an EC_{50} of 1.08 ± 0.09 μM and an EC_{90} of 2.69 ± 0.47 μM, as revealed by plaque assay. It also inhibited RNA replication with an EC_{50} = 7.41 ± 1.09 μM, as depicted by *Renilla luciferase* reporter assay and qRT-PCR of intracellular and extracellular vRNA levels. The inhibition of DENV-2 entry starts at a higher concentration of Amodiaquine (10 - 25 μM) compared to the concentration (5 μM) needed to inhibit the infectivity, when administered post-infection.
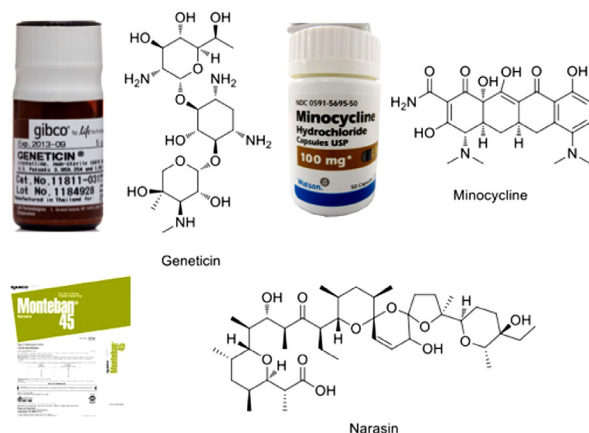
Figure 12. Antibiotic drugs repurposed as DENV inhibitors
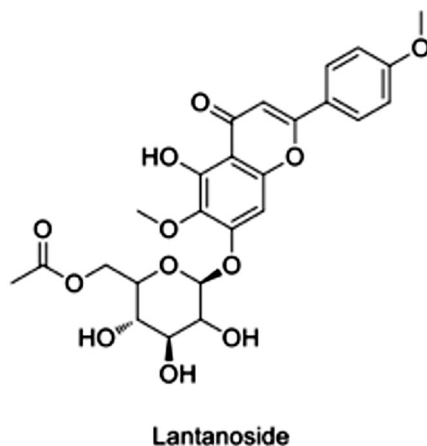
Figure 13. Antiarrhythmic drugs repurposed as DENV inhibitors

4.3. Antidiabetic agents

4.3.1. Castanospermine

Castanospermine is a natural alkaloid that acts as ER-glucosidase I inhibitor in cells and also reduces infection of enveloped RNA and DNA viruses. It is active *in vitro* against influenza virus, cytomegalovirus, HIV-1 and DENV-1 and *in vivo* against Herpes Simplex Virus and Rauscher Murine Leukemia Virus (46). Glucosidase inhibitors such as Castanospermine and Deoxynojirimycin showed inhibitory activity against DENV-1 interfering with the folding of the structural proteins prM and E, which intervene in a crucial step of virion secretion. *In vitro* assays demonstrated that Castanospermine is able to reduce the infection of all the 4 serotypes of DENV. Castanospermine inhibited the production of infectious DENV-2 with an IC_{50} of 85.7 μM in the Huh-7 human hepatoma cell line and with an IC_{50} of 1 μM in BHK-21 cells. It was able to prevent the mortality of A/J mice after treatment for

10 days at a dose ranging from 10 to 250 mg/kg of body weight per day, with 85% of survival rate (P < 0.0001) compared to the vehicle. In contrast, doses higher than 250 mg/kg generated weight loss, diarrhea and other side effects, probably due to gastrointestinal toxicity (46). This means that Castanospermine has a remarkable antiviral activity against DENV, most likely due to interference wiht the folding stage of the prM and E proteins that became unstable and not suitable for envelope glycoprotein processing (47).

4.3.2. Celgosivir

α-Glucosidase I is an enzyme that plays a critical role in viral maturation by initiating the processing of the N-linked oligosaccharides of viral envelope glycoproteins (48). In this context, Celgosivir, an oral prodrug of alpha-glucosidase I inhibitor Castanospermine, was deeply studied for the treatment of infections caused by enveloped viruses like HCV (49). Celgosivir was also screened against the

four serotypes of DENV, showing an EC_{50} ranging from 0.22 to 0.68 µM. Fluorescence microscopy analysis suggested that the antiviral activity of this compound may be due, in part, to misfolding and accumulation of DENV non-structural protein 1 (NS1) in the ER and, in part, to the modulation of the host unfolded protein response (UPR) responsible for pro-survival/pro-apoptotic protein levels (50–51). Celgosivir showed 88% reduction of viremia *in vivo* (AG129 infected mouse model) after treatment for three days at a dose of 75 mg/kg (52). Another *in vivo* experiment conducted in mice infected with a lethal dose confirmed the efficacy of this compound, resulting in enhanced survival, reduced viremia and robust immune response, as reflected by serum cytokine analysis, even when a post-infection treatment was used (51). After *in vivo* and *in vitro* experiments, Celgosivir was evaluated in a clinical trial named CLADEN (53). Patients received an initial dose of Celgosivir of 400 mg within 6 h. Later, they received maintenance doses of 200 mg every 12 h for a total of nine doses (total dose 2.0 g). In this trial, Celgosivir showed a modest antiviral effect, like a reduced NS1 protein amount in the serum of the patients compared to placebo, but its activity was strain and cell type dependent. Failure of the trial and, more generally, failure of Celgosivir in humans could be due to virus strain and cell type dependency of this drug. Celgosivir, in fact, showed good efficacy in a mouse model study, in which the compound was administered on the day of infection, but was less effective in another *in vivo* analysis, when it was administered during the peak of viremia requiring higher doses to reduce the viremia level (52).

4.3.3. Deoxynojirimycin

Deoxynojirimycin (DNJ) is a natural iminosugar extracted from Mulberry leaves, which is endowed with alpha-glucosidase inhibitory activity and also showed antidiabetic and antiviral effects (54). Two derivatives of DNJ, N-hydroxyethyl-DNJ (miglitol) and N-butyl-DNJ (miglustat), are currently used to cure diabetes and Gaucher's disease, respectively. These different activities are due to glycosidase inhibition, even if some derivatives showed a different therapeutic application by interacting with sugar receptors or chaperone deficient enzymes, like in cystic fibrosis and in lysosomal storage disorders. Moreover, iminosugars are able to inhibit ER budding viruses by inhibiting ER-glucosidase enzyme. In this context, it was found that a derivative of DNJ, the N-nonyl-DNJ (NN-DNJ), was able to suppress DENV-2 infection in a dose-dependent manner. Inhibition of the viral replication step is probably due to a reduction in the secretion of the glycoproteins E and NS1 and in production of viral RNA, as revealed by fluorogenic RT-PCR. In addition, interaction between prM, E, and NS1 with ER chaperone calnexin, is affected by NN-DNJ, meaning that calnexin participates in the folding step of those

glycoproteins (55). NN-DNJ was also screened in an AG129 mouse model showing good oral bioavailability and a potent reduction (93%) of viremia at a dose of 75 mg/kg twice a day for 3 days.

4.3.4. CM-10–18

CM-10–18 is an iminosugar that inhibits host cellular α-glucosidases I and II. These enzymes sequentially remove the three terminal glucoses in the N-linked oligosaccharides of viral envelope glycoproteins; from this process derives the proper folding of viral glycoproteins and the subsequent assembly of enveloped viruses like DENV. CM-10–18 and a similar derivative, CM-9–78, showed a satisfactory *in vitro* inhibitory activity against DENV replication and reduced the viremia level of DENV in an *in vivo* mouse model. These two derivatives showed a favourable pharmacokinetic profile at a dose around 100 mg/kg in rats. Moreover, combination of CM-10–18 and RBV showed an enhanced inhibitory activity against DENV, compared to the antiviral activity of RBV alone (56).

4.4. Antihistamine drugs

4.4.1. Cromolyn, montelukast, ketotifene

Cromolyn is a mast cell (MC) stabilizer that prevents the release of inflammatory mediators like histamine. It is used in the treatment of allergic rhinitis, asthma, allergic conjunctivitis and ulcerative colitis. Montelukast is a leukotriene receptor antagonist used for the treatment of asthma, seasonal allergies and bronchospasm. Ketotifene is a noncompetitive H^1-antihistamine and MC stabilizer. It is mainly used to treat allergic conjunctivitis, itchy red eyes caused by allergies and to prevent asthma attacks. These three derivatives act as MC modulators: Montelukast inhibits MC activity, blocking mediators like the leukotriens, instead Cromolyn and Ketotifen stabilize MC membrane, avoiding the release of mediators of the inflammation. The use of these drugs resulted in a reduction of vascular leakage in the wild type and immune-compromised mouse models of DENV (57). DENV infection, in fact, triggers MC activation with subsequent release of vasoactive products, including chymase, that promotes vascular leakage. This means that there is a direct correlation between MC activation and DENV disease severity and that MC are potential therapeutic targets to prevent DENV-induced vasculopathy. In addition, the MC-specific mediator chymase can be considered a biomarker of dengue fever (DF) and dengue hemorrhagic fever (DHF) (58).

MC modulators could be highly effective in secondary heterologous infections (or maternal transmission of antibodies to infants) that lead to

severe DENV disease. The immunological memory of MC due to Fc receptors and their binding to multiple types of antibodies generates an additional mechanism of release of vasoactive inflammatory products during the infection that can contribute to DENV pathogenesis and vascular leakage.

4.5. Anticancer drugs

4.5.1. Dasatinib and AZD0530 (Saracatinib)

Dasatinib is a pyrimidine-thiazole derivative endowed with inhibitor activity against Src and Bcr-Abl kinases. It is used in the treatment of chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia resistant to Imatinib (59). AZD-0530 (Saracatinib) is another kinase inhibitor active against Src and Bcr-Abl (60a,b). Originally it was developed for the treatment of cancer, but it did not show sufficient efficacy in cancer patients and, subsequently, was evaluated against Alzheimer's disease. Dasatinib showed elevated cellular activity against Bcr-Abl and Src-family kinases, but also low selectivity, inhibiting platelet derived growth factor receptor (PDGFR), stem cell factor (c-kit), ephrin A2 (EPHA2) receptor tyrosine kinases and p38 MAP kinase with considerably high potency. AZD-0530, instead, is a more specific inhibitor endowed with higher activity against Src than Bcr-Abl (about 10 times). These two derivatives were identified as DENV inhibitors after the development of a novel immunofluorescence screening, based on the detection of DENV envelope proteins (61). Dasatinib and AZD-0530 were further evaluated on Vero, Huh-7 and C6/36 cells infected by DENV2 at noncytotoxic concentrations of 50 nM-5 µM showing a dose dependent inhibition of the viral infection on all the three cell lines and validating the kinase Src as a potential target for DENV eradication. AZD-0530, being more selective, was also screened against DENV-1, -3 and -4 serotypes, Modoc virus (a murine flavivirus) and Poliovirus 1 (a virus of the picornavirus family) showing a selectivity against the flaviviridae family of pathogens. In addition, AZD-0530 was tested on c-Src siRNA knockdown Huh-7 cells, demonstrating a strong correlation between the antiviral activity and c-Src inhibition. Pretreatment with the two kinase inhibitors did not affect virus infection, meaning that c-Src protein kinase activity may play an important role in a post-entry phase, like viral RNA replication, virion assembly and maturation, or viral egression. After immunofluorescent staining, it was evident that c-Src inhibitors do not affect RNA synthesis or gene expression of the virus but that they reduce the presence of DENV virions in the lumen of the endoplasmatic reticulum. This means that c-Src is important for the budding of the nucleocapsid into the lumen and for the following formation of viral particles.

In another study, AZD-0530 and Dasatinib showed an IC_{90} of 12.2 and 4.7 µM, respectively, and a CC_{90} of 95 and 132 µM on Huh 7 cells infected by DENV2 (62). Pre- and post-infection drug treatment, used to determine the mechanism of action of the two derivatives, led to comparable results meaning that the target of the inhibitors is a host cofactor. Single-cycle reporter virus particle (RVP) system analysis highlighted that viral entry is not affected by the two kinase inhibitors. Northern blot analysis showed that these two compounds inhibited the viral replication by decreasing the accumulation of genomic and subgenomic viral RNAs. Being AZD-0530 and Dasatinib ATP competitive inhibitors of Abl- and Src-family kinases (AFK and SFK), the pharmacological inhibition of these two families was used to determine the mechanism of action of Dasatinib and AZD-0530. Since the shutting down of AFK did not show any effect on DENV-2 replication, it was supposed that one of the 11 members of the SFK family of kinases (Blk, Brk, Fgr, Frk, Fyn, Hck, Lyn, Lck, Src, Srms, and Yes) is responsible for DENV replication. After siRNA mediated depletion of the SFK, it was determined that Fyn is the host target mostly involved in DENV replication and the target of AZD-0530 and Dasatinib. Also, depletion of Lyn and Src had inhibitory effects on DENV-2 replication meaning that Fyn kinase can act in combination with these two kinases during the infection.

4.5.2. Bortezomib

Bortezomib is a dipeptide boronic acid analogue that inhibits the proteasome, an enzyme complex that regulates protein degradation in a controlled fashion and has a central role in the Ubiquitin Proteasome Pathway (UPP) (63). It was approved in the U.S. for the treatment of multiple myeloma and mantle cell lymphoma (64). This anticancer agent has been recently considered a potential Dengue virus inhibitor after the discovery that UPP is required for DENV egress (65). To demonstrate that Bortezomib is able to inhibit DENV egress at a cellular level, it was evaluated on primary monocytes (66), finding a CC_{50} above 1 µM. Plaque assays on cells infected by each of the four DENV serotypes showed that the genome is maintained also after treatment, but the infectivity of the pathogen is reduced and, at higher drug concentrations, even abolished. In addition, viral replication was inhibited with an EC_{50} of less than 20 nM by pretreatment with Bortezomib in clinical isolates of the four serotypes from primary monocytes. Treatment of mice caused reduction of the number of infected cells in the red pulp of the spleen indicating that proteasome inhibition limits the egress of the virus and consequent spreading of the infection.

Comparison between treated and control animals showed also a reduced degree of

thrombocytopenia and plasma leakage due to the use of Bortezomib that caused reduction of inflammatory response, mast cell activation and levels of circulating TNFα. All these data clearly indicate that Bortezomib inhibits DENV-2 replication and the subsequent proinflammatory response *in vivo*. Although the potential therapeutic application of this drug in DENV infected patients should be definitely evaluated in clinical trials, the idea of preventing viral egress through proteasome inhibition represents a valuable therapeutic strategy.

4.6. Antipsycotic drugs

4.6.1. Prochlorperazine

Prochlorperazine, a dopamine D2 receptor (D2R) antagonist, is an antipsychotic agent with a phenothiazine structure, mainly used in the treatment of nausea, vomiting, and vertigo. It showed potent *in vitro* and *in vivo* antiviral activity against DENV infection. It is able to block viral infection by targeting the binding and entry stages of DENV through D2R- and clathrin-associated mechanisms (67). Prochlorperazine, at the noncytotoxic concentration of 30 µM, inhibits protein expression and viral progeny in HEK293T DENV-2 infected cells, with an EC_{50} of 88 nM. The same behaviour was also shown by Prochlorperazine dimaleate solution, but with a slightly higher EC_{50} of 137 nM, probably due to the different solubility of the formulation (Novamin was used before). The drug could interfere with early steps of infection, since DENV-2 production is reduced in a dose dependent fashion, when a treatment during viral adsorption was evaluated.

Chlatrin distribution and DENV entry through clathrin-mediated endocytosis are affected by Prochloperazine. Treatments with 20 and 30 µM of the drug, influenced the distribution of clathrin in mock and HEK293T infected cells. Prochlorperazine, in fact, led to colocalization of DENV-2 and clathrin on the cellular surface. To determine, if the effect of Prochloperazine could be also due to the interference with another early step of DENV life cycle, the influence of dopamine D2 receptors in DENV replication was also evaluated. It was found that Dopamine D2 receptors are important for the entry phase of the pathogen: in fact, Prochlorperazine exerted antiviral activity only in cells expressing Dopamine D2 receptors (N18, shLacZ-N18, shD2R-D2RL-N18, and shD2R-D2RS-N18) and not in D2-knockdown cells (shD2R-N18). Prochloperazine was also evaluated as dimaleate and novamin formulations *in vivo* in immunocompromised *Stat1*$^{-/-}$ mice, using different doses, times of addition and administration. It was concluded that the drug has a dose dependent protective effect improving mice survival and reducing the viremia levels. Prochloperazine proved to have two beneficial effects, inhibiting DENV replication and relieving symptoms associated with the infection, like headache, nausea and vomiting. The mechanism of action of this drug involves two cellular components instead of viral proteins, preventing possible insurgence of drug-resistance. All these data make Prochloperazine an interesting agent for therapeutic and prophylactic treatment of DENV infected patients.

4.7. Antiparasite drugs

4.7.1. Ivermectin

Ivermectin is a mixture of two macrolides derived from Streptomyces avermitilis, called avermectins. Ivermectin increases permeability and hyperpolarization of nerve and muscle cells by binding glutamate-gated chloride channel (68). It is a broad spectrum antiparasitic agent active against microfilariae of Onchocerca Volvulus (not in the adult form), mainly used in humans for the treatment of onchocerciasis, scabies and lice (69a-c). Ivermectin was proposed as DENV inhibitor by *in silico* docking studies on an unexploited site, the region of ssRNA access involved in helicase activity of the NS3 protein (70). The crystal of the NS3 helicase domain of Kunjin virus (another virus belonging to the flaviviridae family) was used as a model for the docking, since the structure of the NS3 DENV-helicase complex was not available. Ivermectin inhibited DENV helicase with an IC_{50} of 500±70 nM in a FRET-based helicase assay and did not inhibit ATPase activity of the helicase domain or polymerase activity of DENV at a concentration of 1 µM. Mutant helicases were prepared to determine important interactions of Ivermectin with this enzyme: Ivermectin was not effective on the prepared mutants, indicating that mutations in the catalytic site are detrimental for Ivermectin binding to the helicase, as predicted from structural analysis. In CPE reduction assays using Vero-B cells, Ivermectin showed an EC_{50} > 1 µM and in qRT-PCR analysis an EC_{50} of 0.7 µM. Furthermore, helicase kinetics and inhibition tests showed that the presence of RNA was needed for Ivermectin to effectively bind the protein. Ivermectin acts as an uncompetitive inhibitor and it is more effective in the first 14 h after treatment. Ivermectin is also able to inhibit DENV infection by interfering with the interaction of NS5 with the Importin superfamily of proteins (71). Importin, and more specifically the heterodimer Imp*α/ß1*, is important for recognition and subsequent movement of proteins between nucleus and cytoplasm. Using an AlphaScreen protein-protein binding assay, it was shown that Ivermectin is able to inhibit the interaction of Imp*α/ß1* and NS5 with an IC_{50} of 17 µM, but not the interaction of NS5 with Imp*ß1* (IC_{50}>22mM). This result demonstrates also that this drug is selective for one of the many nuclear import mechanisms. In addition, at a concentration of 25 µM, it completely inhibits the virus production in Vero cells. These data demonstrated that inhibitors of the nuclear

import could be considered potent and selective antiviral agents.

4.7.2. Suramin

Suramin is a symmetrical polyanionic polysulfonated naphthylamine derived from urea, used in the treatment of African Trypanosomiasis and in association with diethylcarbamazine to kill adult Onchocerca. It also showed high antineoplastic activity and antiviral effects against DENV, HIV, Chikungunya and other viruses (72). Suramin was identified as a promising hit compound from a screening protocol aimed at the discovery of novel DENV inhibitors (73). A novel fluorescence-based assay that monitors helicase activity of DENV NS3 protein was used to screen a library of 1600 small molecules obtained from the Experimental Therapeutics Centre (ETC, Singapore). After the screening, a dose response experiment performed at concentrations ranging from 0.1 to 300 μM confirmed that Suramin was the best NS3 inhibitor. It showed 100% inhibition of NS3 helicase in the primary screen and a dose dependent inhibition with an IC_{50} of 0.4 μM. Suramin showed a non-competitive mechanism of inhibition with a Ki of 0.75 ± 0.03 μM meaning that this polyanionic derivatite is able to bind to the free form of the enzyme. In addition, Suramin also inhibits NS3 activity of different DENV serotypes like DENV-3 (IC_{50} = 0.6 – 0.8 μM) and DENV-4 (IC_{50} = 0.8 μM). These *in vitro* analyses indicate that Suramin is a good drug-candidate for *in vivo* studies.

4.7.3. Nitazoxanide A

Nitazoxanide is the precursor of the active derivative Tizoxanide, a metabolite that is endowed with *in vitro* and *in vivo* activity against a variety of microorganisms, including a broad range of protozoa and helminths (74). Nitazoxanide was originally commercialized as an antiprotozoal drug, but later it was found to be active also against gram positive and negative bacteria, Mycobacterium Tubercolosis and a broad range of DNA and RNA viruses including DENV (75). Nitazoxanide was able to inhibit DENV-2 with an IC_{50} of 0.1 μg/ml in Vero cells with an SI of 10 (76). It was most effective when cells were treated 2 h post-infection, with a decrease in antiviral activity when cells were treated at 5 and 10 h post-infection. Nitazoxanide and its active metabolite Tizoxanide are active against a vast panel of pathogens with low cytotoxicity and represent therefore highly promising candidates for future development into clinical candidates.

4.8. Anticholesteremic drugs

4.8.1. Lovastatin

Lovastatin is a fungal metabolite of the statins' family that is commonly used as anticholesterol agent for its lipid lowering activity. This activity is due to inhibition of the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA Reductase), a rate-controlling enzyme in the methabolic pathway of cholesterol. Statins showed also anti-inflammatory and antiviral properties making them promising agents for the treatment of viral infections (77). Moreover, the discovery that the presence of cholesterol affects replication of flaviviruses opened the opportunity to use these compounds against pathogens like DENV (78a,b). In fact, Lovastatin showed inhibitory activity of DENV replication in epithelial cells (VERO cells) with an IC_{50} of 38.4 μM, a CC_{50} of 57.2 μM and an SI of 1.4, and on endothelial cells (HEMC-1) with an IC_{50} of 11.9 μM, a CC_{50} of 53.6 μM and an SI of 4.5 (79). Furthermore, a time-of-addition (TOA) study highlighted that Lovastatin, if added before viral inoculation, is able to decrease viral entry by reducing membrane cholesterol. If the drug is added after virus inoculation, inhibition of prenylation probably affects the transport from ER to the Golgi apparatus thereby preventing the secretion of newly formed virus particles. In an *in vivo* analysis, Lovastatin was administered to different groups of AG129 infected mice at a dose of 200 mg/kg/day once, twice and three times before and after virus inoculation. Similar results were obtained when considering survival rate (2, 2.5 days), but differences can be appreciated when considering viremia levels. Virus titer in serum decreased (21.8%) in the group treated before virus inoculation and increased (21.7%) in the group treated after DENV inoculation, suggesting that time-of-addition is more relevant in animal models than in cell models (80). Finally, a randomized double-blind placebo-controlled trial aimed at investigating a short course therapy with Lovastatin was conducted in a group of adult patients infected by DENV (81). Around 300 patients were chosen for the trial. The treatment was performed for 5 days at a dose of 80 mg. Unfortunately, the trial did not show clear evidences of beneficial effects on any of the clinical manifestations of the disease or on Dengue viremia (82).

4.9. Steroid drugs

4.9.1. Dexamethasone

Dexamethasone is an anti-inflammatory and immunosuppressant agent. It is used in the treatment of rheumatic problems, skin diseases, severe allergies, asthma, chronic obstructive lung disease, croup, brain swelling and, in combination with antibiotics, for the treatment of tuberculosis. Considering that steroids are used to increase platelet count in idiopathic thrombocytopenic purpura and that thrombocytopenia in Dengue Fever (DF) is probably due to a peripheral destruction of antibody coated platelets and bone marrow suppression, the use of this class of drugs in the treatment of DENV infection was almost obliged. For this reason, a study with high intravenous dosage

of dexamethasone was conducted to increase platelet count in acute stage of Dengue Fever with thrombocytopenia. For this study, 127 patients were recruited. Two groups were formed: a study group received an intravenous dose of dexamethasone of 8 mg initially, followed by doses of 4 mg every 8 h for 4 days. The second group received only IV fluids and antipyretics, whenever it was indicated. During the four days of treatment, platelet count was measured daily. At the end of the analysis, a slight increase of platelet counts was observed in both groups. The conclusion of this study was, that high doses of dexamethasone were not effective in avoiding thrombocytopenia and consequent Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) in the acute stage of Dengue Fever (83).

4.9.2. Prednisolone

Prednisolone is a synthetic glucocorticoid that derives from the naturally occurring steroid Cortisol. It is the active metabolite of Prednisone and it is used to treat a variety of inflammatory and autoimmune conditions and some types of cancers. Like other corticosteroids, it is able to reduce typical inflammatory and hemorrhagic conditions due to DENV infection like capillary permeability, hemorrhagic shock, thrombocytopenia and bleeding (84). In order to evaluate the efficacy of Prednisolone against DENV infections, a randomized placebo-controlled blinded trial of low (0.5. mg/kg) and high doses (2 mg/kg) therapy was conducted on young patients for 3 days (85). 225 patients participated in the trial, but unfortunately, the use of this corticosteroid during early acute phase of viral infection could not be associated with reduction in the development of shock or other recognized complications due to DENV infection. The reason for the lack of efficacy of Prednisolone treatment is probably due to the fact that it was not given at the right time and/or at the adequate dose in order to alleviate the infection that generates altered capillary permeability, thrombocytopenia and hemostatic derangements (86).

4.10. Antibiotic drugs

4.10.1. Geneticin

Geneticin is an analog of the aminoglycoside antibiotic Neomycin, which interferes with the function of 80S ribosomes and with protein synthesis in eukaryotic cells. It was shown to inhibit DENV-2 replication and translation, showing higher activity for this virus compared to other pathogens like YFV (87). In addition, Geneticin's structural analogues like gentamicin, kanamycin and guanidylated geneticin had no effect on DENV infection highlighting also a structural specificity. Geneticin showed an EC_{50} value of 3.0±0.4 µg/ml in BHK cells, inhibiting the cytopathic effect (CPE) induced by DENV-2 infection. It also inhibited viral yield with an EC_{50} of 2.0±0.1 µg/ml and an EC_{90} of 20±2 µg/ml. Moreover, considering the CC_{50} of 165±5 µg/ml, Geneticin has a SI of 66 in BHK cells infected by Dengue virus. Virus-induced plaque formation can also be arrested with Geneticin (25 µg/ml) treatment. RT-qPCR demonstrated that viral RNA synthesis is inhibited 12 h after Geneticin treatment by 40%, meaning that it does not block the early events of DENV infection. For the effect on translation of viral proteins, viral E protein was used as a marker of DENV-2 translation. Treatment with Geneticin inhibited the formation of E protein by 80%. In addition, the absence of additional E protein bands in the treated sample suggests that this antibiotic does not affect the processing of DENV-2 E protein. Unfortunately, the mechanism of antiviral activity of Geneticin is still unclear, but it is plausible that it inhibits viral translation, resulting in a decrease of viral RNA synthesis. Alternatively, it is also possible that Geneticin inhibits viral RNA folding, preventing accumulation of viral RNA and consequently in an overall decrease in viral proteins.

4.10.2. Narasin

Narasin is an antibacterial and antibiotic derivative used for the treatment of coccidia in chicken, active also against fungi and bacteria (88). A high-throughput cell-based screening on a highly purified natural products library (Biomol) was conducted to identify potential antiviral agents against DENV (89). A library of 502 natural products was analyzed. After an initial screening, 30 compounds were selected using a criterium of 40% of inhibition. A second round of assays, using 50–75% of inhibition against DENV-2 was performed to identify 4 derivatives. Out of these four, Narasin showed an IC_{50} of 0.39 µM and an SI of 2.5. It was tested (plaque assay quantification) on mononuclear phagocytic primary cells at increasing concentrations showing an IC_{50} of 0.32 µM and a low cytotoxicity. Narasin is active against all four DENV serotypes with IC_{50} values of 0.65, 0.39, 0.44, 0.05 µM against DENV-1, -2, -3, -4, respectively, in HuH-7 infected cells. Time-of-addition studies revealed that the drug is not effective at an early stage of viral infection, but probably during the release of viral genome into the cytoplasm. (RT)-PCR analysis showed that viral RNA replication is not affected by Narasin. Western blot assays highlighted that expression level of DENV E and NS5 viral proteins is reduced at the early time point of 12 h. Finally, ultrastructural imaging conducted after Narasin treatment revealed the lack of adverse effects on cellular morphology. Narasin resulted to be a promising hit for further development as a DENV drug candidate.

4.10.3. Minocycline

Minocycline is a semi-synthetic derivative of a naturally occurring tetracycline with antibiotic activity. For its anti-inflammatory and immunomodulatory effects it has been widely used in the treatment of infections of the urinary tract, rheumatoid arthritis and acne (90a,b). From evaluation of the antiviral effects of three antibiotics, lomefloxacin, netilmicin, and minocycline, the latter emerged as the most effective in reducing DENV replication (91). In addition, this antiviral activity was confirmed in all four DENV serotypes. Treatment with minocycline affects viral RNA synthesis, expression of intracellular enveloped proteins and production of virions. This antibiotic showed a CC_{50} of 1482 µM on HepG2 cells and reduced the phosphorylation of host cofactors like extracellular signal-regulated kinase1/2 (ERK1/2) useful for expression of IFN-induced antiviral genes (e.g. OAS1, OAS3). Upregulation of Interferon and antiviral genes after Minocycline treatment was also observed in HepG2 DENV-2 infected cells. The activity of Minocycline against Dengue virus infection is a good result, although the mechanism of ERK1/2 inhibition and IFN-α upregulation should be better determined.

4.11. Antiarrhythmic drugs

4.11.1. Lanatoside C

Lanantoside C is a cardioactive glycoside able to inhibit the Na^+-K^+-ATPase pump and approved by the FDA for treatment of two common types of arrhythmia: atrial fibrillation and paroxysmal supraventricular tachycardia. Recently, it was demonstrated that Lanantoside C is endowed with antiviral activity against negative-strand RNA viruses including influenza virus, vesicular stomatitis virus and Newcastle disease virus (92). Lanatoside C was identified after a high-throughput screening of the US Drug Collection library as a potent DENV inhibitor candidate (93). The effect of this cardiac glycoside on DENV-2, and later on other serotypes, was evaluated by production of infectious virus particles and viral RNA as well as viral protein expression. Lanatoside C was non-cytotoxic at concentrations of 1.0 µM in HuH-7, U937 and HUVEC cells and at concentrations of 0.1 µM in U937 cells. In addition, it inhibited DENV-2 replication in HuH-7 cells with an IC_{50} of 0.19 µM, a CC_{50} of 5.48 µM and a relatively high SI of 28.84. In U937 cells, IC_{50}, CC_{50} and SI were 0.03 µM, 0.47 µM and 15.67. In HUVEC cells, IC_{50} was 0.30 µM and the CC_{50} was not calculated. DENV-2 replication was inhibited exclusively after addition of Lanatoside C at or before 24 h post infection but not after 48 h, indicating that the drug targets post-entry stages like the translation of viral proteins, viral replication complex assembly, and viral RNA synthesis.

Lanatoside C showed a good antiviral effect and low cytotoxicity *in vitro*. The mechanism of action is not well understood, but it seems to be connected to cellular factors rather than viral proteins. Further biological investigation and *in vivo* studies may demonstrate the value of this drug in the antiviral scenario.

5. CONCLUSIONS

Since the classical drug-discovery approach is often a long, stiff and costly process, the identification of new indications for already existing drugs (drug repositioning) could improve and enhance the actual number of new medicines that reach the market. Drug repositioning aims at fulfilling the need of new drugs for a given disease, especially for emerging diseases or for those still without treatment, thanks to the accelerated drug discovery route on which this approach is based. In fact, pharmacokinetic, pre-clinical and, often, clinical data are already available for marketed drugs (or any other drugs dropped in late stage of development), allowing to bypass early drug discovery stages and thus saving time and money. Until now, many molecules approved or investigated for their activity on a specific target (primary action), have been successfully repositioned for new indications (secondary action). Especially for diseases like DENV, an effective "shortcut" to new marketable drugs could represent a life-saving deal for thousands of people since this disease is endemic in the poorest regions of the globe. In this review, we have reported several molecules, belonging to different pharmacological classes of drugs, which have been repurposed for treatment of Dengue Virus infection and related diseases. Even if some of the drugs reviewed herein did not demonstrate a substantial effectiveness against DENV infection, a consistent number of them could be considered a suitable and advanced starting point to develop new treatments, demonstrating the importance of the "repurposing" technique in finding new treatments for this emerging infectious disease.

6. ACKNOWLEDGEMENTS

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Abbreviations: AFK: Ab1-family kinases, ALT: Alanine Aminotransferase, AP: Alkaline Phosphatase, AST: Aspartate Aminotransferase, ATA: Aurintricarboxylic Acid, BV: Banzi Virus, BVDV: Bovine Viral Diarrhea Virus, C: Capsid, CPE: Cytophatic Effect , DENV: Dengue Virus, DF: Dengue Fever, DHF: Dengue Hemorrahagic Fever, DNJ: Deoxynojirimycin, DSS: Dengue Shock Syndrome, D2R: Dopamine D2 Receptor, E: Envelope, EPHA2: Ephrin A2, ER: Endoplasmatic Reticulum, ERK1/2: Extracellular Signal-regulated Kinase 1/2., HCV: Hepatitis C Virus, HIV-1: Immunodeficiency Virus 1, HMG-CoA Reductase: 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase, HTS: High-Throughput Screening, IMPDH: Inosine Monosphate Dehydrogenase, JEV: Japanese Encephalitis Virus, MC: Mast Cell, MPA: Mycophenolic Acid, MTase: Methyltransferase, NN-DNJ: N-nonyl- Deoxynojirimycin, NS: Non-Structural, PBM: Peripheral Blood Mononuclear Cell, PDGFR: Platelet Derived Growth Factor Receptor, PrM: Pre-Membrane, RdRP: RNA-dependent RNA Polymerase, RBV: Ribavarine, RSV: Respiratory Syncytial Virus, RVP: Reporter Virus Particle, SFK: Src-family Kinases, TOA: Time-of-addiction, UPP: Ubiquitin Proteasome Pathway, UPR: Unfolded Protein Response, WHO: World Health Organization, WNC: West Nile Virus, YFV: Yellow Fever Virus

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