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Present perspectives on flaviviral chemotherapy

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Flaviviruses are a group of arboviruses under the family of Flaviviridae. Flaviviruses are global pathogens, some of which cause neurotrophic disorders from mild febrile illness to lethal encephalitis while others cause severe hemorrhagic fever. These viruses are endemic to many parts of the world and periodic outbreaks take thousands of lives. With globalization of the world accompanied by gradual shift in global climate, the viruses are emerging in newer areas and we are in a point of exigency to decipher new drugs to combat these emerging threats. Although extensive research has taken place and vaccines for few of these viral diseases are available, still no chemotherapeutic agent has been found to deliver an absolute cure to flaviviral infections. It is important to assess the present status of flavivirus drug research and delve deep into the avenues of flavivirus drug discovery. This review outlines the chemotherapeutic agents explored till date, and highlights perspectives on a possible future breakthrough – at least to ameliorate if not to abrogate the diseases.

Flaviviruses are a group of arboviruses belonging to the Flaviviridae family, and in the genus Flavivirus. The other family members are Pestiviruses and Hepaciviruses [1]. The genus is made up of 70 small positive-stranded RNA viruses with genome size ~11 kb. The genome RNA represents the only messenger RNA in the infected cells and encodes three structural proteins (C, capsid protein; Pre M, the membrane precursor protein; and envelope E protein) and seven nonstructural (NS) proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5) [2,3]. The virus is transmitted by mosquitoes of *Culex* spp. and *Aedes* spp. and also by ticks. The flaviviral diseases are now global in nature and cause severe and often fatal diseases in humans and agriculturally important animals, ranging from lethal encephalitis caused by Japanese Encephalitis Virus (JEV) to hemorrhagic fevers from dengue virus. Some of the important diseases spread by the flaviviruses are West Nile encephalitis (WNE), Murray valley encephalitis (MVE), St. Louis encephalitis (SLE), Japanese encephalitis (JE), dengue fever, Yellow fever (YF), among others.

The diseases caused by the flaviviruses are emerging in new areas and populations, or increasing in frequency and geographic distribution [4] (Fig. 1). For example, WNE previously unknown in North America made its first appearance in New York in 1999. After the introduction of West Nile Virus (WNV) in the USA, a total of 23,925 cases of WNV infection and 946 deaths has been reported to Centers for Disease Control and Prevention (CDC) between 1999 and 2006, with the largest ever WNE outbreaks occurring in the summers of 2002 and 2003. There are numerous other flaviviral diseases such as dengue fever and JE, that have spread throughout the world especially much of Asia and Oceania [5]. At present, dengue fever is one of the most important mosquito-borne diseases of the world with 3 billion people at risk in endemic tropical areas [4]. The cause of the sudden emergence of the flaviviruses lies with rapid globalization of the world and with the change in the global climatic condition. In fact, according to a report published by Centers for Disease Control and Prevention (see: http://www.cdc.gov/ncidod/eid/vol6no4/rappole.htm), researchers believe that WNV could have entered the West via a number of routes such as travel by infected humans, introduction of infected birds and unintentional introduction of virus-bearing mosquitoes. Scientists are blaming dramatic global environmental change on account of industrialization, deforestation and population explosion to be the cause behind the emergence of previously

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1359-6446/06/$ - see front matter doi:10.1016/j.drudis.2008.04.001

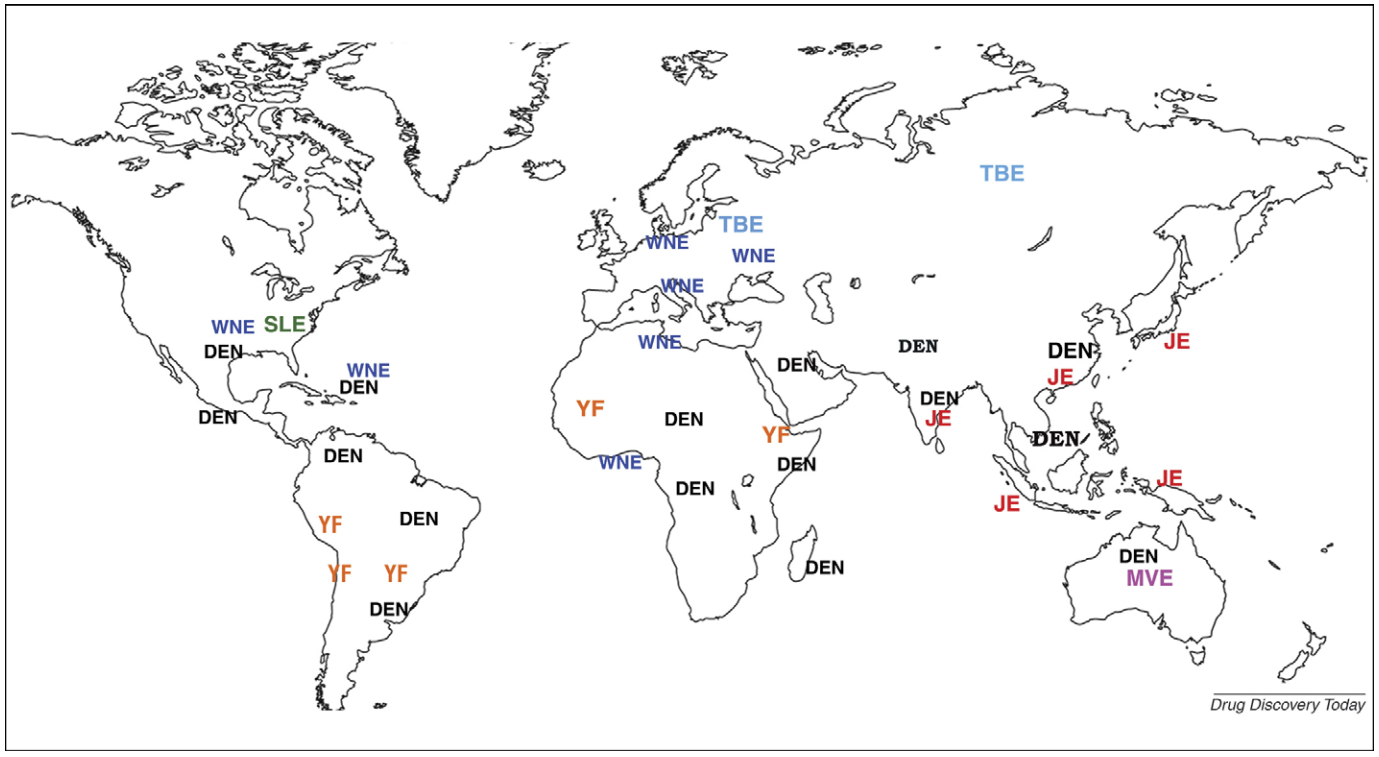

FIGURE 1

The global distribution of dominant or potentially important Flaviviruses. This information is originally adapted from the website of Center for disease Control and Prevention (see: http://www.cdc.gov/). JE, Japanese encephalitis; MVE, Murray valley encephalitis; SLE, St. Louis encephalitis; TBE, tick borne encephalitis; WNE, West Nile encephalitis; YF, yellow fever; DEN, dengue fever.

suppressed infectious diseases (see: http://www.unep.org/Documents.Multilingual/Default.asp?DocumentID=424&ArticleID=4728&l=en).

The re-emergence of flaviviral diseases has left us in dire need for an antiflaviviral drug. Although vaccines of few of the flaviviruses, for example, JEV and YFV, are available there are still no chemotherapeutic drugs available for the treatment of the flaviviral diseases. The main difficulty in chemotherapeutic antiviral drug discovery is that, after studying millions of compounds, only a handful of the drugs show desired activity and unfortunately many compounds out of these few have toxic side effects, or are poorly absorbed. Therefore, eventually less than 1% of the compounds studied are developed into drugs. However, drug research all over the world has led to the discovery of many compounds with antiflaviviral activity. This review not only outlines some of the compounds that have generated excitement and hold prospects of being developed into flaviviral drug but it also focuses on the present status of flaviviral drug research.

Pathogenesis

Myriad of factors govern the severity of flaviviral diseases. Thus, for JEV the failure of the host to produce antibodies to the virus is associated with an increased likelihood that the disease will become lethal [6]. Crossing the blood–brain barrier is an important factor for the increased pathogenesis and clinical outcome of the neurotropic viral infection [7]. Reports suggest macrophages could serve as a reservoir for WNV, spreading the virus from the periphery to the CNS [8,9]. Other studies have shown that WNV is capable of entering the CNS via anterograde axonal transport [10], while JEV virions binding to the endothelial surface of the CNS are internalized by endocytosis [11]. Neurologic diseases like JEV typically develop in patients after an incubation period of 5–15 days. The manifestation of the disease depends on which part of the nervous system is affected and includes symptoms like reduced level of consciousness, seizures, flaccid paralysis resembling that of poliomyelitis, and parkinsonian movement disorder [12]. Symptoms of typical classic dengue usually start with fever within four to seven days of infection and include high fever, severe headache, retro-orbital pain, severe joint and muscle pain, nausea, vomiting and rash. Symptoms of dengue hemorrhagic fever include all of the symptoms of classic dengue fever plus marked damage to blood and lymph vessels, bleeding from the nose, gums or under the skin, causing purplish bruises (see: http://www.who.int/mediacentre/factsheets/fs117/en/).

Glimpse at research in the recent past

The chemotherapy against viral infections can be developed by two principally different approaches. In the first approach the virus coded function is blocked and in the second approach the cellular functions needed for the viral multiplication are blocked. The second approach has the problem that it also hampers normal cellular function, but it is advantageous in the way that the therapy is active against all the viruses of the same genus, and emergence of resistance against this type of chemotherapy is rare.

Since the early 1990s, research all over the world unearthed various compounds, both synthetic and natural, having inhibitory effects on different flaviviruses. Here, we discuss some of the important compounds discovered in the recent past in this field.

Drugs inhibiting viral replication

Research carried out as early as 1977 has shown that compounds such as 2-deoxy D-glucose (2dDG) and 3 diazuridine in combination have an inhibitory effect on the multiplication of JEV. The compound 2dDG is a competitor of glucose, while 3 diazuridine is a competitor of uridine. When administered individually on BHK21 cell cultures they showed no substantial antiviral activity, but in combination they have prominent antiviral effects even in a dose much lower than that which induces cellular cytotoxicity [13].

Chemotherapeutic agents targeting the viral helicase were developed in the form of the nucleoside analogue HMC-HO4, which inhibits the WNV helicase (NS3) activity and WNV replication in Vero cells at half maximal inhibitory concentration (IC_{50}) of 30 μM [14]. Later, certain ring-expanded nucleoside analogues, and halogenated benzimidazoles and benzotriazoles, were shown to inhibit WNV–NS3-mediated unwinding of viral substrates [15].

A particularly important compound discovered in antiflaviviral drug research history is ribavirin. Ribavirin (1-β-D-ribofuranosyl-1H-1, 2,4-triazole-3 carboxamide) was initially synthesized as a guanosine analogue in 1970. It was immediately recognized to possess activity against several RNA and DNA viruses. Ribavirin was first approved for use in humans for the treatment of severe Respiratory Syncytial Virus. Ribavirin is a nucleoside analogue, whose mechanism of action includes the inhibition of inosine monophosphate dehydrogenase (IMPDH), required for *de novo* guanine synthesis, which in turn is required for viral replication. Besides the inhibition of IMPDH, ribavirin was reported to possess other antiviral mechanisms of action such as functioning as a mutagen to cause error catastrophe or to serve as an RNA cap analogue [16]. Since the identification of Hepatitis C virus (HCV), ribavirin in combination with IFN-α became a routine treatment for this disease [17], but efficacy was further enhanced when IFN-α was substituted by peg-IFN-α2b [18,19]. In the realms of flavivirus chemotherapy, ribavirin was known to be an anti-WNV drug (see: http://www.patentstorm.us/patents/6946125.html). Later, in a separate study in 2004, ribavirin was evaluated for the treatment of YFV in a hamster model of the disease [20]. Ribavirin proved effective at an optimum dose of 80 mg/kg body weight. The study showed that survival was 100% when the treatment started on day 1, 2 or 3 post-infection, but it dropped to 87% when administration occurred after the 3rd day post-infection. The histopathology of the liver, spleen and pancreas of the ribavirin treated animals was markedly less than that of the virus-only infected placebo group; also the treated animals have less depletion of lymphoid cells indicating the immunoprotective role of ribavirin.

Other nucleoside triphosphate inhibitors reported as having anti-WNV activity include compounds like mycophenolic acid, 6-azauridine, 6-azauridine acetate, pyrazofurin, 2-thio-azauridine and cyclopentenylcytosine [21]. All these compounds inhibit oritidine monophosphate decarboxylase (OMPDC), except mycophenolic acid [22], which like ribavirin inhibits IMPDH.

Brequinar is an antiviral agent shown to inhibit YFV and DENV in *in vitro* studies. This drug acts as an inhibitor of cellular dihydroorotate dehydrogenase [21]. Moreover, compounds like diethyldithiocarbamate (DDTC) in combination with IFN-α2b have been shown to prevent mortality in 25% of mice given the lethal dose of JEV [23,24].

Recent research showed that Triaryl Pyrazoline {[5-(4-chlorophenyl)-3-thiophen-2-yl-4, 5-dihydro-pyrazol-1-yl]-phenyl-methionine} inhibits flavivirus replication in cell culture. The compound inhibited an epidemic strain of WNV without detectable cytotoxicity. Besides WNV, the compound also inhibited other strains of flaviviruses like DENV, St. Louis encephalitis virus (SLEV) and YFV. This compound is thought to act not by preventing viral entry or assembly but by inhibiting RNA replication [25]. Overall, Triaryl Pyrazoline exerts broad spectrum antiflaviviral activity and this novel inhibitor could be developed for potential treatment in flavivirus infection.

Interesting research on the analysis of the influence of rare earth ions on the entry of flaviviruses WNV and Uganda S virus has shown that 30 s treatment with lanthanide ions such as La^{3+}, Ce^{3+}, Pr^{3+} or Nd^{3+} changed cellular chemistry into a state in which cells no longer supported viral replication [26]. This change occurs in cells treated before, during or after infection and the change does not interfere with host cell replication. But this change required repeated treatment of the flavivirus infected cells every 24 h with the appropriate lanthanide ion. Actually, lanthanide ion treatment blocked the ability of the host cell to support replication of flavivirus RNA. An important aspect of this finding was, unlike interferon (IFN), lanthanide ion treatment was effective even in the presence of Actinomycin D and cellular RNA translation remained unchanged. More in-depth research on this topic could pave the way for the development of a general chemotherapeutic against flavivirus.

A report on the study of the inhibitory potential of the crude extract of *Quercus lusitanica* seeds on the replication of DENV2 generated excitement because it inhibited viral replication in C6/36 cells (cloned cells of *Aedes* larvae) in a dose-dependent fashion when assessed employing a virus inhibition assay. The extract at its maximum nontoxic concentration of 0.25 mg/ml completely inhibited 10–1000 tissue culture infectious dose 50 ($TCID_{50}$) of virus as indicated by the absence of any cytopathic effect. The report also highlighted the ability of the compound to down-regulate NS1 protein expression and this ability could play a part in the antiviral potential of the *Q. lusitanica* seed extract [27].

Drugs inhibiting viral entry in host

Several inhibitors of flavivirus entry in the host have been recognized. These are mainly sulfated polysaccharides and polyoxotungstates. A study reported the ability of six polyoxotungstates to inhibit dengue fever virus-2 (DENV2) at IC_{50} values of 0.45–36.8 μM, without any apparent toxicity in Vero cells [28]. Investigation also revealed that sulfated polysaccharides inhibit DENV2 adsorption and internalization in human and monkey cells at IC_{50} ~1.0 μg/ml [29,30]. Sulfated galactomannans by contrast renders protection to the mice when co-inoculated with YFV, but failed to do so in the three-day post-infection model [31].

Drugs inhibiting viral protein folding

Castanospermine is a water-soluble natural alkaloid derived from black bean or Moreton Bay chestnut tree. Investigation has revealed that this compound acts as an α glucosidase inhibitor and inhibits dengue fever virus-1 (DENV1) *in vitro* by disrupting the folding of the structural proteins PrM and E. At the turn of the millennium, separate *in vivo* and *in vitro* studies showed that other

α-glucosidase inhibitors like deoxynojirimycin, *N*-nonyl-deoxynojirimycin and 6-*O*-butanoylcatanospermine inhibited DENV1, DENV2 and JEV [32,33]. Later studies on the effect of castanospermine on all serotypes of dengue fever virus (DENV), WNV and YFV have shown that the compound successfully inhibited all DENV serotypes but failed to inhibit YFV and WNV [34]. Further studies are needed on these glucosidase inhibitors to develop these for clinical use.

Drugs having anti-inflammatory and antiapoptotic action

A notable breakthrough in antiflaviviral drug research is the discovery of minocycline as an antiviral drug. Minocycline is a member of the broad spectrum tetracycline antibiotics, but recent research programs have also shown it to have antiviral properties. Minocycline is a semi-synthetic second-generation tetracycline that exerts anti-inflammatory and antiapoptotic effects that are completely separate from its antimicrobial action. A study on the effect of minocycline on WNV pathogenesis on different CNS-derived cell types such as primary human retinal pigment epithelial cells and a T98G human glioma cell line has shown that minocycline exerts its strongest anti-WNV activity by inhibiting the WNV-induced apoptosis and WNV replication [35]. The study highlighted that nontoxic minocycline concentrations, which can be achieved in human tissues, significantly reduced WNV titers in all the cell types tested. The study also showed that minocycline inhibited WNV induced apoptosis and suppressed virus-induced activation of C-Jun N-terminal kinase (JNK) and its target C-Jun. The next significant research on minocycline as an antiflaviviral drug was an *in vivo* study on the effect of minocycline on the GP78 strain of JEV [36]. The study was done on adult mice and was the first of its kind where it was shown that neuronal apoptosis, microglial activation, active caspase activity, proinflammatory mediators and viral titer were markedly decreased in minocycline-treated mice dosed on the 9th day post-infection. Thus, minocycline is a promising candidate to be developed as a broad-spectrum antiflaviviral drug.

Another naturally occurring compound discovered to have antiflaviviral activity and the potential to be developed as an antiflaviviral drug is rosmarinic acid (RA). RA, a phenolic compound found in various *Labiatae* herbs, possesses several anti-inflammatory and antioxidative properties. This study was conducted *in vivo* where the effect of RA was tested on the mice treated with GP78 strain JEV and it showed that RA reduces viral replication in murine brain and also ameliorates the secondary inflammation resulting from microglial activation [37].

Current research of interest in antiflaviviral drug discovery is the identification of the novel compound arctigenin as a drug for the treatment of JEV, one of the most significant causes of viral encephalitis worldwide. Arctigenin is a naturally occurring phenylpropanoid dibenzylbutyrolactone lignan with antioxidant, anti-inflammatory, neuroprotective and antiviral properties. The study reported the therapeutic effects of arctigenin on a murine model of experimental JEV, where treatment with arctigenin confers complete protection to the animal from JEV infection by markedly decreasing JEV-induced neuronal apoptosis, microglial activation, active caspase activity and induction of proinflammatory mediators in the brains of arctigenin-treated mice [38]. The study went further to claim that Arctigenin may act directly on brain cells, because a neuronal cell line was also salvaged from JEV-induced cell death. Thus, arctigenin seems to show substantial promise for being developed into a new therapeutic agent.

Drugs with unknown mechanism of action

Research has shown the compound 2-amino-8-(B-D-ribofuronosyl) imidazo [1,2-*a*]-*s*-triazine-4-one (ZX-2401) was found to be a broad-spectrum antiviral agent, capable of inhibiting at least five members of the Flaviviridae family in cell culture with minimal cytotoxicity. This compound inhibited YFV, DENV and WNV with half maximal effective concentrations (EC_{50}) of 10, 10 and 3 μg/ml, respectively. The half cytocidal concentration (CC_{50}) in this experiment was greater than 100 μg/ml. Furthermore, ZX-2401 exhibited synergistic antiviral activity in combination with IFN in tissue culture [39]. However, further study is needed to develop this compound as a future drug.

As a mode of prevention of the spread of flavivirus infection, methylene blue can act as a safe and cost-effective way to neutralize the WNV infection *in vitro* and avoid severe illness and mortality in transfusion patients [40]. In this study, methylene blue and light, inactivated three primary WNV isolates (obtained from years 1999, 2002 and 2003) and prevented mortality in a murine model of WNV infection.

Present perspectives

Flavivirus drug research is moving at an expeditious pace and we are hopeful that a dependable chemotherapeutic agent will soon be at hand to abrogate the flaviviral diseases. Researchers are now considering all molecular aspects of the flaviviruses and targeting their different proteins for drug development.

Earlier, we discussed the antiflaviviral activity of ribavirin but this drug has a disadvantage of causing hemolytic anemia. In view of overcoming the problem, researchers have now developed ribavirin analogues, a newer category of IMPDH inhibitors. One of the compounds viramidine, a liver targeting prodrug of ribavirin, has demonstrated significant antiviral activity and erythrocyte sparing properties and currently is in Phase 3 clinical trials. Another notable compound is merimepodip, and is undergoing Phase 2 trials [41].

In the light of modern technical advancement, researchers are now focusing on structure based drug design techniques, which connect crystallography and NMR, computational advances in docking algorithms and virtual screening and traditional techniques of high-throughput screening (HTS) with combinatorial chemistry to increase the efficiency and speed of drug discovery.

Efforts are being channeled into the design of a future drug against the NS2B and NS3 nonstructural proteins of the flaviviruses. These nonstructural proteins form a protease complex that is necessary for crucial viral polyprotein processing. Recent research has been carried out to elucidate, the crucial physicochemical and biochemical properties of the proteases using chromogenic and flurogenic substrates. The study also compared proteases from seven different flaviviral enzymes and also analyzed optimal proteolytic condition [42]. Such a study will bear fruit in forms of a successful drug targeting this protease complex. Further, scientists are trying to target the viral capping enzyme. It

is worth mentioning the recent patent [43] in the synthesis of guanylyltransferase and methyltransferases, which will be a model of flaviviral capping enzyme. Cap is a unique structure found at the 5′ end of viral and cellular mRNA. The cap is needed for the stability of the mRNA and it binds to the ribosome during translation. The capping enzyme of flaviviruses remained an enigma and was not characterized, so the present synthesized enzymes may act as a model of the flavivirus capping enzyme and help researchers to design a chemotherapeutic drug to inhibit the capping enzyme of this genus in a unique manner.

Other studies using crystallography-based models of NS3 substrate interaction compounds containing a single guanidino group that could potentially interact with the S1 pocket of NS3 are identified. It has been suggested by modeling studies that compound containing a single guanidino arm inhibited WNV and DENV helicase activity by forming hydrogen bonds with an active serine residue in the S1 pocket [44]. Although all the NS2b and NS3 inhibitors identified so far have generated plenty of excitement, extensive research is still needed to improve the specificity and potency of the compounds to be used clinically as drugs.

The envelope protein E of the flaviviruses, responsible for receptor binding and membrane fusion, is also a very promising target for future drug design. The protein forms an icosahedral cage-like structure of homodimers that completely cover the surface of the mature virions. The detailed molecular structure of the E protein is revealed and chemotherapeutic drugs and neutralizing antibodies are being developed for future antiflaviviral medicine. In fact, researchers have successfully identified and tested a small molecule that inhibits DENV fusion mechanism. In the study compounds were identified by *in silico* docking of a compound library against the DENV E protein. In the process, a lead compound has been identified as having no mammalian cytotoxicity with an IC_{50} of 1 μM. This compound has shown the promise of being developed into an antiflaviviral drug in the future [45]. More interestingly, the conserved structure of the flavivirus E protein may help the application of this future drug well beyond DENV treatment.

Studies were also carried out on NS5, the largest protein encoded by flavivirus genome and most conserved of the flaviviral proteins. This protein has the RNA-dependent RNA polymerase activity and thus is a key element for the flaviviral replication. Scientists at Merck have reported that a polymerase inhibitor from the nucleoside class (7-deaza-2′-C-methyl adenosine) and one of its analogues has activity on DENV2 and other flaviviruses [46]. Studies have elucidated the crystal structure of enzymatically active and inactive NS5 domains at 3.0 and 2.35 Å resolution, respectively [46]. Studies are also conducted on the DENV RNA polymerase-5 to understand its biochemical properties, subcellular localization and regulations. Some of these properties may be exploited for our quest for a DENV curing drug. In fact a report of an *in vitro* study shows that NS5 protein of DENV2 needed to be exported from the nucleus to the cytoplasm for viral RNA replication, and is mediated by the exportin1 protein CRM1 [47]. The study went further to show that the administration of the CRM1 specific inhibitor leptomycin B alters the kinetics of viral production. This report clearly places the nuclear export system of NS5 under the radar of antiflaviviral chemotherapy.

Advances in our knowledge of the molecular biology of the flaviviruses and construction of the flavivirus replicon system have paved the way for HTS of flaviviral inhibitors. One of the most promising approaches in drug development is taking place in an elaborate collaborative project using the mega-computing power of World Community Grid (see: http://www.worldcommunitygrid.org/projects_showcase/dddt/viewDddtMain.do). The researchers are conducting extensive calculations to identify new drug-like molecules that will bind very tightly to the viral proteases and inhibit their activity. Thus, the search is on for compounds against WNV, YFV, DENV and HCV. The binding calculations combine rigorous mean-field molecular dynamics algorithms, with the Autodock virtual docking program used to predict accurately how tightly the small molecules bind to the flavivirus proteases. Autodock will fit millions of different small molecules of flavivirus proteases, so that the best fitting molecule can be identified. The preliminarily identified molecule will then be further analyzed with CHARMM a molecular dynamic program. At last, the novel compounds predicted to be high affinity inhibitors would be tested in laboratory assays for antiviral activity. The scale and depth of this project carry enough promise for a significant breakthrough in flaviviral drug research.

Finally, it is important to mention that the discovery of antiviral activity in natural compounds like RA, Arctigenin and *Q.lusitanica* extract opens a new vista – the huge reservoir of the medicinal plants and their extracts available to us, and further study is still to be done to exploit the potential that these natural products may possess to be developed into antiflaviviral drugs. Studies revealed that different classes of compounds like flavonoids, alkaloids, polysaccharides, thiophenes, terpenoids, lectins, lignans, among others, isolated from various plants have different antiviral properties and targets of viral inhibition [48]. Thus, extensive research needs to be channeled toward this arsenal still unexplored.

Conclusion

With cutting edge technologies of biomedical science at hand, the future bears hope for a breakthrough in flaviviral drug discovery. The scientific community all over the world has been awakened by the recent re-emergence of the flaviviral diseases, as it has now become a global problem. Although drug discovery may be achieved with considerable use of technology and sophistication, it is the responsibility of the government of different countries and WHO to extend the fruit of this research to the masses at an affordable cost. A considerable percentage of the flaviviral outbreaks occur in tropical countries like India, Indonesia and Brazil, among others, where large masses are living below the poverty line. Therefore, it is the responsibility of the scientific community to find a drug that could reach the unprivileged masses and only then will our quest for an antiflaviviral chemotherapy will be successful.

Acknowledgements

The work in the author's laboratory is funded by the grant from the Department of Biotechnology (Award#BT/PR/5799/MED/14/698/2005 and BT/PR8682/Med/14/1273/2007), and the Council of Scientific and Industrial Research ((27(0173)/07/EMR-II), Government of India. We are grateful to Abhishek Ghosh for his thoughtful comments.

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