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(54) Title: AGENTS THAT INHIBIT FLAVIVIRUS REPLICATION AND USES THEREOF

(57) Abstract: The present invention provides compounds with activity against a variety of flaviviruses, uses thereof, and methods for identifying such compounds.

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AGENTS THAT INHIBIT FLAVIVIRUS REPLICATION AND USES THEREOF

CONTINUING APPLICATION DATA

This application claims the benefit of U.S. Provisional Application Serial No. 60/632,749, filed December 2, 2004, which is incorporated by reference herein.

GOVERNMENT FUNDING

The present invention was made with government support under Grant No. U54 AI057156, awarded by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, P42296LS, awarded by the Department of Defense, and DAAD19-01-1-0322 and DAAD19-01-1-0361, awarded by the Defense Advanced Research Projects, Department of Defense. The Government may have certain rights in this invention.

BACKGROUND

Arthropod-borne flaviviruses such as dengue virus, West Nile virus, yellow fever virus, Japanese encephalitis virus and tick borne encephalitis virus cause significant public health problems worldwide and pose threats as agents of biowarfare and bioterrorism. These viruses are endemic in many areas of the world and are emerging in other areas. The recent establishment of West Nile virus in North America demonstrates the long-term, damaging consequences that can arise from the introduction of a flavivirus disease agent into a previously non-endemic area. Although the broad vertebrate and invertebrate host specificity of West Nile virus may have predisposed North America to this incursion, multiple mosquito vectors for dengue virus, Japanese encephalitis virus, and yellow fever virus are present in the United States, indicating that these diseases could also become established, and yellow fever was established in many cities in the United States in the late 1800s. Furthermore, the fear and public anxiety that West Nile virus outbreaks generated, the speed of its spread

across the United States, and the association of its spread with organ donation and transfusion demonstrate that flaviviruses can terrorize a civilian population even without intentional malicious introduction. Dengue includes a spectrum of illnesses caused by infection with one of four serotypes of dengue virus that occur in many tropical and subtropical regions. The distribution of dengue has expanded over the last thirty years to include more than one hundred countries (World Health Organization (WHO), "Dengue/dengue haemorrhagic fever," Weekly Epidemiological Record 75:193-196, 2000). Based on the extent of dengue virus infections (approximately one hundred million per year) and the high incidence of severe dengue disease (approximately 500,000 per year), dengue virus is considered to be the most threatening arthropod-borne virus (WHO, 2000). Although dengue virus causes the greatest number of cases of human disease of any flavivirus, Japanese encephalitis and yellow fever are also important diseases, affecting hundreds of thousands of people each year. In addition, approximately 10,000 confirmed cases of West Nile virus infection occurred in the United States in 2003. Furthermore, there is evidence that tick borne encephalitis virus and yellow fever virus, two flaviviruses that can display case-fatality rates of up to 50%, have been developed for use as bioweapons.

There are currently no effective chemotherapies for treating flaviviral infections and licensed vaccines exist only for yellow fever, Japanese encephalitis and tick-borne encephalitis viruses (Barrett, "Current status of flavivirus vaccines," Ann. NY Acad. Sci. 952:262-271, 2001). Current prevention is largely focused on control of the mosquito vector (Gibbons and Vaughn, BMJ 324(7353):1563-6, 2002). Thus, there is a need for cost effective chemotherapeutic agents for the prevention and control of flavivirus infections, including dengue virus and West Nile virus. Broad-spectrum antivirals that inhibit infection by dengue virus and other flaviviruses could combat these bioterrorist agents and significantly improve National Security and global public health.

SUMMARY OF THE INVENTION

In one aspect, the present invention includes a method of treating a flavivirus infection including administering 1,3-Bis(4-nitrophenyl)urea, a

derivative of 1,3-Bis(4-nitrophenyl)urea, 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate.

In another aspect, the present invention includes a method of treating a flavivirus infection including administering 1,3-Bis(4-nitrophenyl)urea, or a derivative thereof.

In another aspect, the present invention includes a method of treating a flavivirus infection including administering 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative thereof.

In another aspect, the present invention includes a method of treating a flavivirus infection in a subject, the method including administering 1,3-Bis(4-nitrophenyl)urea, or a derivative thereof, to a subject infected with a flavivirus in an amount effective to inhibit replication of the flavivirus.

In another aspect, the present invention includes a method of treating a flavivirus infection in a subject, the method including administering 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative thereof, to a subject infected with a flavivirus in an amount effective to inhibit replication of the flavivirus.

In another aspect, the present invention includes a method of preventing infection of a subject with a flavivirus, the method including administering 1,3-Bis(4-nitrophenyl)urea, or a derivative thereof, to the subject prior to exposure to a flavivirus.

In another aspect, the present invention includes a method of preventing infection of a subject with a flavivirus, the method including administering 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative thereof, to the subject prior to exposure to a flavivirus.

In another aspect, the present invention includes a method of reducing the severity of symptoms associated with a flavivirus infection, the method including administering 1,3-Bis(4-nitrophenyl)urea, or a derivative thereof, to the subject prior to infection with a flavivirus. In some embodiments, the flavivirus infection is an infection with a dengue virus and wherein development of dengue hemorrhagic fever and/or dengue shock syndrome is prevented.

In another aspect, the present invention includes a method of reducing the severity of symptoms associated with a flavivirus infection, the method including administering 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative thereof, to the subject prior to infection with a flavivirus. In some embodiments, the method of claim 8 or 9 wherein the flavivirus infection is an infection with a dengue virus and wherein development of dengue hemorrhagic fever and/or dengue shock syndrome is prevented.

In another aspect, the present invention includes a method of reducing the severity of the symptoms associated with a flavivirus infection, the method including administering 1,3-Bis(4-nitrophenyl)urea, or a derivative thereof, to the subject after infection with a flavivirus. In some embodiments, the flavivirus infection is an infection with a dengue virus and wherein development of dengue hemorrhagic fever and/or dengue shock syndrome is prevented.

In another aspect, the present invention includes a method of reducing the severity of the symptoms associated with a flavivirus infection, the method including administering 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative thereof, to the subject after infection with a flavivirus. In some embodiments, the flavivirus infection is an infection with a dengue virus and wherein development of dengue hemorrhagic fever and/or dengue shock syndrome is prevented.

In another aspect, the present invention includes a method of inhibiting the replication of a flavivirus in a cell, the method including contacting cells with 1,3-Bis(4-nitrophenyl)urea, or a derivative thereof.

In another aspect, the present invention includes a method of inhibiting the replication of a flavivirus in a cell, the method including contacting cells with 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative thereof.

In another aspect, the present invention includes a method of identifying an agent suitable for the treatment or prevention of a flavivirus infection, the method including contacting cells with a flavivirus and an agent that is a derivative of 1,3-Bis(4-nitrophenyl)urea, wherein the production of a decreased

flavivirus titer indicates the agent is suitable for the treatment or prevention of a flavivirus infection.

In another aspect, the present invention includes a method of identifying an agent suitable for the treatment or prevention of a flavivirus infection, the method including contacting cells with a flavivirus and an agent that is a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, wherein the production of a decreased flavivirus titer indicates the agent is suitable for the treatment or prevention of a flavivirus infection.

In another aspect, the present invention includes a method of identifying an agent suitable for the treatment or prevention of a flavivirus infection, the method including contacting a flavivirus protease with an agent that is a derivative of 1,3-Bis(4-nitrophenyl)urea, wherein an inhibition of the protease activity of the flavivirus protease indicates the agent is suitable for the treatment or prevention of a flavivirus infection.

In another aspect, the present invention includes a method of identifying an agent suitable for the treatment or prevention of a flavivirus infection, the method including contacting a flavivirus protease with an agent that is a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, wherein an inhibition of the protease activity of the flavivirus protease indicates the agent is suitable for the treatment or prevention of a flavivirus infection.

In another aspect, the present invention includes a method of identifying an agent suitable for the treatment or prevention of a dengue virus infection, the method including contacting the NS3 serine protease of a dengue virus with an agent that is a derivative of 1,3-Bis(4-nitrophenyl)urea, wherein an inhibition of the serine protease activity of the NS3 serine protease of a dengue virus indicates the agent is suitable for the treatment or prevention of a dengue virus infection.

In another aspect, the present invention includes a method of identifying an agent suitable for the treatment or prevention of a dengue virus infection, the method including contacting the NS3 serine protease of a dengue virus with an agent that is a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, wherein an inhibition of the serine protease activity of the

NS3 serine protease of a dengue virus indicates the agent is suitable for the treatment or prevention of a dengue virus infection.

In some embodiments of the methods of the resent invention, the flavivirus is selected from the group consisting of dengue 1 virus, dengue 2 virus, dengue 3 virus, dengue 4 virus, West Nile virus, Yellow Fever virus, Japanese encephalitis virus, Murray valley fever virus, Yakose virus, Apoi virus, Rio Bravo virus, Modoc virus, Deer tick virus, Langat virus, Powassan virus, Tick-borne encephalitis virus, and Hepatitis C virus.

In some embodiments of the methods of the present invention, the dengue virus is selected from the group consisting of dengue 1 virus, dengue 2 virus, dengue 3 virus, and dengue 4 virus.

In another aspect, the present invention includes a method of treating a flavivirus infection including administering 1,3-Bis(4-nitrophenyl)urea, or a derivative thereof, and 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative thereof.

In another aspect, the present invention includes a method of preventing infection with a flavivirus, the method including administering 1,3-Bis(4-nitrophenyl)urea, or a derivative thereof, and 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative thereof.

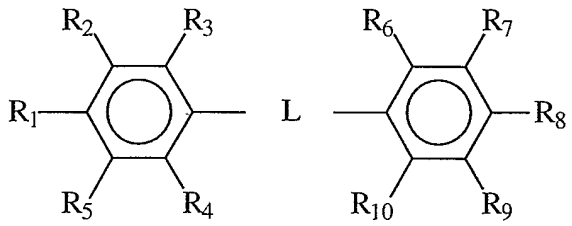
In another aspect, the present invention includes a method of reducing the severity of the symptoms associated a flavivirus infection, the method including administering 1,3-Bis(4-nitrophenyl)urea, or a derivative thereof, and 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative thereof.

In another aspect, the present invention includes a method of inhibiting the replication of a flavivirus in a cell, the method including contacting cells with 1,3-Bis(4-nitrophenyl)urea, or a derivative thereof, and 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative thereof.

In some embodiments of the methods of the present invention, the method further includes administering one or more additional antiviral agents.

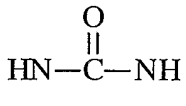
In some embodiments of the methods of the present invention, the method further includes contacting the cells, flavivirus protease, or NS3 serine protease with or more additional antiviral agents.

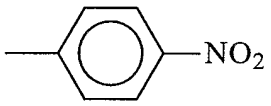
In another aspect, the present invention includes a derivative of 1,3-Bis(4-nitrophenyl)urea, wherein the derivative inhibits the replication of a flavivirus in cell culture. In some embodiments, the derivative of 1,3-Bis(4-nitrophenyl)urea may be an symmetrical urea derivative. In some embodiments, the derivative of 1,3-Bis(4-nitrophenyl)urea may be an symmetrical urea derivative. In some embodiments, the derivative of 1,3-Bis(4-nitrophenyl)urea may have the structure:



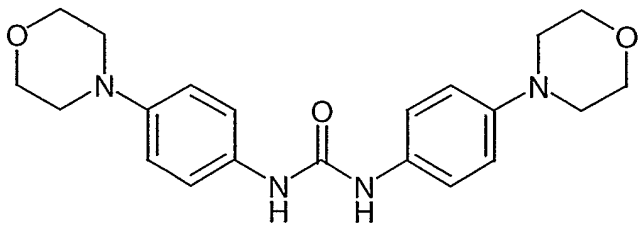
wherein L is a divalent linking group;

wherein R^1, R^2, R^3, R^4, R^5, R^6, R^7, R^8, R^9, and R^{10} are independently selected from H, F, an alkyne, an alkene, a ketone, an aldehyde, an ester or an amine; and

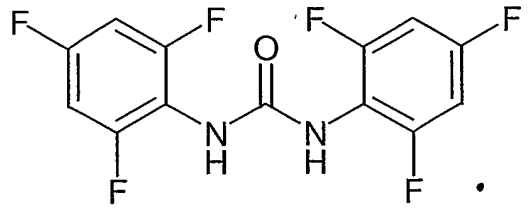
with the proviso that when L is a  moiety,

then R^1 and R^8 are not both a  moiety.

In another aspect, the present invention includes a compound having the formula:



In another aspect, the present invention includes a compound having the formula:



In another aspect, the present invention includes a derivative of 1,3-Bis(4-nitrophenyl)urea, wherein the derivative inhibits the replication of a dengue virus in cell culture.

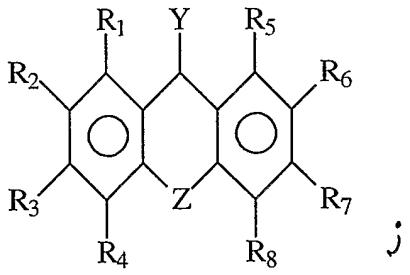
In another aspect, the present invention includes a derivative of 1,3-Bis(4-nitrophenyl)urea, wherein the derivative inhibits the *in vivo* replication of a flavivirus.

In another aspect, the present invention includes a derivative of 1,3-Bis(4-nitrophenyl)urea, wherein the derivative inhibits the *in vivo* replication of a dengue virus.

In another aspect, the present invention includes a derivative of 1,3-Bis(4-nitrophenyl)urea, wherein the derivative inhibits a flavivirus protease.

In another aspect, the present invention includes a derivative of 1,3-Bis(4-nitrophenyl)urea, wherein the derivative inhibits the NS3 serine protease of a dengue virus.

In another aspect, the present invention includes a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, wherein the derivative inhibits the replication of a flavivirus in cell culture. In some embodiments, the derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate may have the structure:

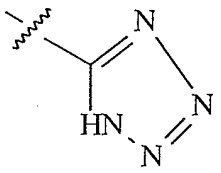


wherein Z is, SO_2, O, or a nitrogen;

wherein Y is, O, H, S, or an imine; and

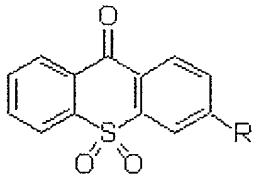
wherein each of R1 to R8 is independently selected from H, a C1-C20 organic group, an amide, or a boronic acid;

with the proviso that when Z is SO_2 and Y is O, then R7 can not be a



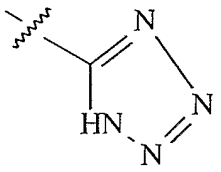
moiety.

In some embodiments, the derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate may have the structure:



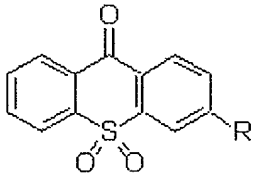
wherein R is H, a C1-C20 organic group, an amide, or a boronic acid;

with the proviso that R is not a



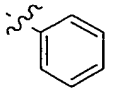
moiety.

In another aspect, the present invention includes a compound having the structure:

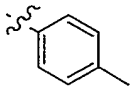


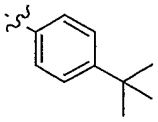
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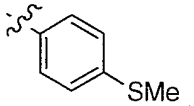
wherein R is selected from:

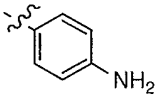


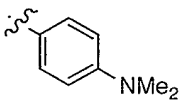
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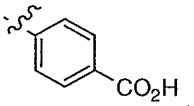
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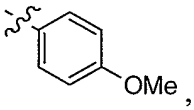
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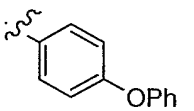
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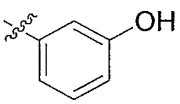
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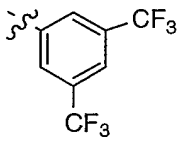
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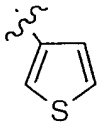
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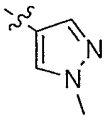
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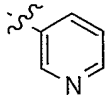
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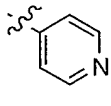
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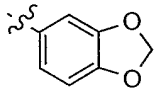
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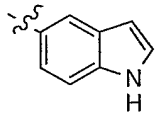

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In another aspect, the present invention includes a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, wherein the derivative inhibits the replication of a dengue virus in cell culture.

In another aspect, the present invention includes a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, wherein the derivative inhibits the *in vivo* replication of a flavivirus.

In another aspect, the present invention includes a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, wherein the derivative inhibits the *in vivo* replication of a dengue virus.

In another aspect, the present invention includes a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, wherein the derivative inhibits a flavivirus protease.

In another aspect, the present invention includes a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, wherein the derivative inhibits the NS3 serine protease of a dengue virus.

In some embodiments of the present invention, the flavivirus may be dengue 1 virus, dengue 2 virus, dengue 3 virus, dengue 4 virus, West Nile virus, Yellow Fever virus, Japanese encephalitis virus, Murray valley fever virus, Yakose virus, Apoi virus, Rio Bravo virus, Modoc virus, Deer tick virus, Langat virus, Powassan virus, Tick-borne encephalitis virus, or Hepatitis C virus.

In some embodiments of the present invention, the dengue virus may be dengue 1 virus, dengue 2 virus, dengue 3 virus, or dengue 4 virus.

In another aspect, the present invention includes a composition including a derivative of 1,3-Bis(4-nitrophenyl)urea and a pharmaceutically acceptable carrier. In some embodiments, the composition may further including one or more additional antiviral agents.

In another aspect, the present invention includes a composition including of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative thereof, and a pharmaceutically acceptable carrier. In some embodiments, the composition may further including one or more additional antiviral agents.

In another aspect, the present invention includes a composition including at least two agents and a pharmaceutically acceptable carrier, wherein one agent is selected from the group consisting of 1,3-Bis(4-nitrophenyl)urea, a derivative of 1,3-Bis(4-nitrophenyl)urea, 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, and a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, and wherein a second agent wherein one agent is selected from the group consisting of 1,3-Bis(4-nitrophenyl)urea, a derivative of 1,3-Bis(4-nitrophenyl)urea, 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, and a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate. In some embodiments, the compositions of the present invention may further include one or more additional antiviral agents.

In another aspect, the present invention includes a bivalent agent including 1,3-Bis(4-nitrophenyl)urea or a derivative of 1,3-Bis(4-

nitrophenyl)urea and 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate or a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate.

In another aspect, the present invention includes a bivalent agent including as one aspect 1,3-Bis(4-nitrophenyl)urea, a derivative of 1,3-Bis(4-nitrophenyl)urea, 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate or a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate and as a second aspect an additional antiviral agent.

In another aspect, the present invention includes a bivalent agent including 1,3-Bis(4-nitrophenyl)urea or a derivative of 1,3-Bis(4-nitrophenyl)urea covalently attached to 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate or a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate.

In another aspect, the present invention includes a bivalent agent including 1,3-Bis(4-nitrophenyl)urea, a derivative of 1,3-Bis(4-nitrophenyl)urea, 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate covalently attached to an additiaonl antiviral agent.

In another aspect, the present invention includes a combinatorial library including at least one derivative of 1,3-Bis(4-nitrophenyl)urea.

In another aspect, the present invention includes a combinatorial library including at least one derivative of 3-(1H-tetrazol-5-yl)-9H-thio xanthen-9-one-10,10-dioxide monohydrate.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 presents a phylogenic tree based on sequence similarities of the flavivirus NS3 protein.

Figures 2A-2B presents the functional flavivirus genome organization. Fig. 2A is a schematic diagram of the flavivirus genome showing position of mature forms of the proteins within the open reading frame (ORF), along with cleavage sites ("*s*" represents signal peptidase cleavage sites, "*v*" represents virally encoded NS2B/NS3 cleavage sites, "*f*" represents furin cleavage sites,

and "?" represents cleavage sites of unknown specificity). Fig. 2B is a cartoon representation of a mature viral particle.

Figure 3 is a surface representation of the apo structure of DEN2V NS3 protease, indicating the two spatially distinct binding sites targeted by virtual screening.

Figure 4 shows 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide ("MTT") toxicity assay results. Solubility limits prevented antiviral candidate ARDP0011 from being examined at higher concentrations.

Figure 5 shows slot-blot results for a subset of EUDOC-suggested compounds. Relative antibody signals are shown for days one, two, and three post-DEN2V infection.

Figure 6 presents results from West Nile virus (WNV) replicon assay, demonstrating activity of antiviral candidate ARDP0011 against WNV replicons.

Figure 7 presents the chemical structures of the computer-predicted candidate dengue antivirals ARDP0011 and ARDP0012.

Figure 8 shows representative dengue virus whole cell ELISA (DAWCE) and cytotoxicity (MTT) dose-response curves for antiviral candidate ARDP0011. Experiments were performed using dengue 2 virus and cultured LLC-MK2 cells. Eight and four replicates were used for each data point in the DAWCE and MTT curves, respectively. Goodness-of-fit statistics to standard dose-response curves were approximately 0.76 and 0.64 for the DAWCE and MTT data, respectively. Dashed lines represent 95% confidence limits for the fitted dose-response curves.

Figure 9 shows decreased infectious dengue 2 virus (measured as foci-forming particles) was produced with increased concentration of antiviral candidate ARDP0012. Error bars are from duplicate independent challenge experiments. The dose-response curve fit the data points with a goodness-of-fit R2 of approximately 0.95. The EC50 value interpolated from the fitted dose-response curve is 18 ± 2 nM.

Figure 10 presents trypsin reaction kinetics demonstrating that lead compounds ARDP0011 and ARDP0012 did not interfere with trypsin proteolysis. Reaction kinetics were monitored by increased 405 nm absorbance due to p-nitroanilide product release. Reaction kinetics were for standard

conditions (no inhibitor; solid circles), 115 uM benzamidine ("BZ"; solid triangles), 24 uM ARDP0011 (open squares), and 670 uM ARDP0012 (open diamonds).

Figure 11 presents an overview of synthetic scheme used for initial combinatorial chemical libraries developed around leads ARPD0011 (top) and ARDP0012 (bottom).

Figure 12 presents an overview of the synthetic scheme for the synthesis of ureas and thioxanthones.

Figures 13A-13C present the chemical structures of Derivative 1 and Derivative 2 of 1,3-Bis(4-nitrophenyl)urea. Fig. 13A presents the chemical structure of 1,3-Bis(4-nitrophenyl)urea. Fig. 13B present the chemical structure of Derivative 1 of 1,3-Bis(4-nitrophenyl)urea. Fig. 13C present the chemical structure of Derivative 2 of 1,3-Bis(4-nitrophenyl)urea.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS OF THE INVENTION

A massive-throughput virtual library screen targeting several sites within the dengue virus NS3 protease resulted in the identification of several compounds with activity against a variety of flaviviruses. With the present invention it has been discovered that the molecule 1,3-Bis(4-nitrophenyl)urea (also referred to herein as 1,3-bis(4-nitrophenyl)urea), and derivatives thereof, and the molecule 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, and derivatives thereof, are effective agents for the treatment and prevention of flaviviral infections.

As used herein a flavivirus is a member of the family Flavivirus, including, but not limited to, dengue virus, West Nile virus, yellow fever virus, Japanese encephalitis virus, Kunjin virus, Murray Valley fever virus, Yakose virus, Apoi virus, Rio Bravo virus, Modoc virus, deer tick virus, Langat virus, Powassan virus, tick-borne encephalitis virus, St. Louis encephalitis virus, and Hepatitis C virus. In some embodiments, the flavivrus infection is a dengue virus infection. As used herein, a dengue virus includes, but is not limited to, a dengue 1 virus, a dengue 2 virus, a dengue 3 virus, or a dengue 4 virus. In some

embodiments, the flavivrus infection is a West Nile virus infection. In some embodiments, the flavivrus infection is a Hepatitis C virus infection

Flaviviruses are divided into a number of serogroups based on cross-neutralization tests and genetic analysis (Kuno et al., J. Virol., 72:73-83, 1998). The family consists of approximately seventy viruses, organized into distinct phylogenetic clades (see Fig. 1) (Ryan et al., J. of General Virology 79:947-959, 1998; Gaunt et al., J. General Virology 82: 1867-1876). Flaviviruses include, for example, dengue virus (DENV), West Nile virus (WNV), hepatitis C virus (HCV), yellow fever virus (YFV), Kunjin virus, Murray Valley fever virus, Yakose virus, Apoi virus, Rio Bravo virus, Modoc virus, deer tick virus, Langat virus, Powassan virus, tick-borne encephalitis virus, St. Louis encephalitis virus, and Japanese encephalitis virus (JEV). These mosquito and tick-borne viruses cause significant public health problems worldwide and pose clear and recognized threats as agents of biowarfare and/or bioterrorism. The National Institutes of Health (NIH) lists the following flaviviruses as Category A-C priority pathogens: dengue, West Nile virus, Japanese encephalitis virus, yellow fever virus, and members of the tick-borne encephalitis viruses (TBEVs) which include Russian spring summer encephalitis (RSSE), central European encephalitis (CEE), Kyasanur Forest disease (KFD), and Omsk hemorrhagic fever (OHF) viruses.

Flaviviruses are endemic in many areas of the world and are emerging in others. Mosquitoes are responsible for spreading dengue throughout tropical and subtropical environments, yellow fever virus throughout tropical and subtropical Africa and South America, and Japanese encephalitis virus throughout Asia and Indonesia (Mackenzie, Emerg. Infect. Dis. 5:1-8, 1999). Mosquito-borne West Nile virus is common in parts of Africa, Asia and Europe, and has recently been introduced into the United States (Anderson et al., Science 286:2331-2333, 1999; Lanciotti et al., Science 286:2333-2337, 1999). Tick-borne flaviviruses are found primarily in central and Eastern Europe and the former Soviet Union and exists as three subtypes; Western subtype (commonly referred to as CEE), Siberian subtype, and Far-eastern subtype, the latter two are often collectively called RSSE (Heinz et al., Family Flaviviridae. In Virus Taxonomy, 7th International Committee for the Taxonomy of Viruses, pp. 859-878. Edited by M. H. V. Regenmortel, Fauquet, C. M., Bishop, D. H.

L., Carstens, E., Estes, M. K., Lemon, S., Maniloff, J., Mayo, M. A. McGeogch, D., Pringle, C. R., and Wickner, R. B. San Diego: Academic Press, 2002; Barrett et al., Arboviruses: Alphaviruses, flaviviruses and bunyaviruses. Medical Microbiology, 16th Edition. Eds: D. Greenwood, R.C.B. Slack and J.P. Peutherer, Churchill Livingstone, pp 484-500, 2002).

Flaviviruses are enveloped viruses composed of a nucleocapsid surrounded by a lipid bilayer containing an envelope (E) glycoprotein and a non-glycosylated membrane (M) protein, which is found in infected cells as the glycosylated precursor termed premembrane (prM) (see Fig. 2B). The nucleocapsid consists of the capsid (C) protein and the single-strand RNA genome. The genome contains a long open reading frame that encodes a polyprotein containing the structural and non-structural (NS) viral proteins (Lindenbach et al., Flavivridae: The Viruses and Their Replication; Chapter 20 in Fundamental Virology, Ed. Knipe, D. M.; Howley, P. M.; Lippincott Williams & Wilkins, 2001, pp.589-640).

Flaviviruses contain a single stranded, positive sense RNA genome, encoding three structural proteins (capsid (C), membrane (M), envelope (E)), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (Chambers et al., Annu Rev Microbiol 44:649-688, 1990). The genome is initially translated as a single, large polyprotein precursor. Post-translational cleavages of the viral polyprotein precursor in the cytoplasm of host-infected cells are necessary to produce the separate structural and functional viral proteins that are essential for replication and assembly of new viral progeny. These cleavages are effected by both host enzymes (including signalases and furin) and by a viral protease encoded by the N-terminal third of NS3. This NS3 viral protease, which is a trypsin-like serine protease with a functional catalytic triad (His-Asp-Ser), is essential for viral replication.

Mature viral proteins are released from the polyprotein by co- and post-translational cleavage by viral and cellular proteases; the majority of polyprotein cleavage is accomplished by the viral protease NS3 and its associated NS2B cofactor (see Fig. 2A) (Ryan et al., J. of General Virology 79:947-959, 1998). Due to its critical role in both virion assembly (via release of mature C) and processing of NS proteins for production of the viral replication complex, the NS3 protease has been identified as a key target for

antiviral drug development (Leyssen et al., Clinical Microbiology Reviews 13:67-82, 2000).

Thus, by virtue of its essential function in post-translational processing of the viral polypeptide, flaviviral NS3 protease is a potential target for development of therapeutic agents that prevent viral replication. Precedents for inhibitors of viral proteases being potential antiviral drugs can be found in the success of inhibitors of proteases of the human immunodeficiency viruses (HIV), currently the most effective treatments for humans with HIV/AIDS (Gulick, Clin Microbiol Infect 9:186-193, 2003; Rutenber et al., J. Biol. Chem. 268:15343-1534610, 1993) and in the early promise being shown by inhibitors of the NS3 protease of Hepatitis C virus (Lin et al., J Biol Chem 279:17508-17514, 2004).

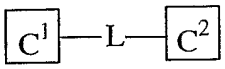
Viral proteases have attracted considerable interest as antiviral targets, since HIV, flaviviruses, alphaviruses, rhinovirus, and adenovirus are dependent on the activity of viral proteases for replication (Strauss, Virol. 1:307-384, 1990). Inhibition of the HIV protease PR has been an effective means of treating HIV infection; between 1995 and 2001 the Food and Drug Administration (FDA) approved six anti-PR peptide analogs as treatments for AIDS (Wlodawer, Annu. Rev. Med. 53:595-614, 2002). In silico methods were successfully employed to help develop several HIV protease inhibitors (Lam et al., Science 263:380-4, 1994). Analogous drug discovery efforts targeting the flavivirus HCV protease have produced an inhibitor reported to reduce viral load in humans (Lamarre et al., Nature 426:186-9, 2003).

With the present invention it has been discovered that the small molecule 1,3-Bis(4-nitrophenyl)urea, and derivatives thereof, and the small molecule 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, and derivatives thereof, are effective agents for the treatment and prevention of flaviviral infections.

As used herein, an "agent" or "compound" includes 1,3-Bis(4-nitrophenyl)urea, a derivative of 1,3-Bis(4-nitrophenyl)urea, 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate.

1,3-Bis(4-nitrophenyl)urea ($(O_2NC_6H_4NH_2)CO$), also known as 4',4"-dinitrocarbanilide or 4,4'-dinitrocarbanilide is commercially available, for example, from Sigma-Aldrich.

Derivatives of 1,3-Bis(4-nitrophenyl)urea include compounds having the general formula represented by Formula I, or a pharmaceutically acceptable salt, solvate, ester, or prodrug thereof. Formula I is as provided below:



Formula I

wherein L is a divalent linking group such as, for example, urea, C(O), S(O), $S(O)_2$, O, an amide, an amide derivative, a nitryl group, or an alcohol; and

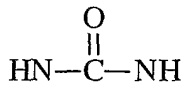
wherein C^1 and C^2 are independently selected organic groups.

As used herein, the term "organic group" is used for the purpose of this invention to mean a hydrocarbon group that is classified as an aliphatic group, cyclic group, or combination of aliphatic and cyclic groups (e.g., alkaryl and aralkyl groups). In the context of the present invention, the term "aliphatic group" means a saturated or unsaturated linear or branched hydrocarbon group. This term is used to encompass alkyl, alkenyl, and alkynyl groups, for example. The term "alkyl group" means a saturated linear or branched monovalent hydrocarbon group including, for example, methyl, ethyl, *n*-propyl, isopropyl, *tert*-butyl, amyl, heptyl, and the like. The term "alkenyl group" means an unsaturated, linear or branched monovalent hydrocarbon group with one or more olefinically unsaturated groups (i.e., carbon-carbon double bonds), such as a vinyl group. The term "alkynyl group" means an unsaturated, linear or branched monovalent hydrocarbon group with one or more carbon-carbon triple bonds. The term "cyclic group" means a closed ring hydrocarbon group that is classified as an alicyclic group, aromatic group, or heterocyclic group. The term "alicyclic group" means a cyclic hydrocarbon group having properties resembling those of aliphatic groups. The term "aromatic group" or "aryl group" means a mono- or polynuclear aromatic hydrocarbon group. The term "heterocyclic group" means a closed ring hydrocarbon in which one or more of the atoms in the ring is an element other than carbon (e.g., nitrogen, oxygen, sulfur, etc.).

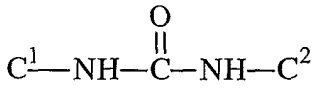
As a means of simplifying the discussion and the recitation of certain terminology used throughout this application, the terms "group" and "moiety" are used to differentiate between chemical species that allow for substitution or that may be substituted and those that do not so allow for substitution or may not be so substituted. Thus, when the term "group" is used to describe a chemical substituent, the described chemical material includes the unsubstituted group and that group with nonperoxidic O, N, S, Si, or F atoms, for example, in the chain as well as carbonyl groups or other conventional substituents. Where the term "moiety" is used to describe a chemical compound or substituent, only an unsubstituted chemical material is intended to be included. For example, the phrase "alkyl group" is intended to include not only pure open chain saturated hydrocarbon alkyl substituents, such as methyl, ethyl, propyl, *tert*-butyl, and the like, but also alkyl substituents bearing further substituents known in the art, such as hydroxy, alkoxy, alkylsulfonyl, halogen atoms, cyano, nitro, amino, carboxyl, etc. Thus, "alkyl group" includes ether groups, haloalkyls, nitroalkyls, carboxyalkyls, hydroxyalkyls, sulfoalkyls, etc. On the other hand, the phrase "alkyl moiety" is limited to the inclusion of only pure open chain saturated hydrocarbon alkyl substituents, such as methyl, ethyl, propyl, *tert*-butyl, and the like.

In a given embodiment, C^1 and C^2 may be either symmetrical or asymmetrical. In some embodiments, either of C^1 or C^2 may be absent.

In some embodiments, L has the formula



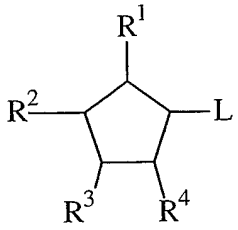
and derivatives have the formula:



Each of C^1 and C^2 may independently be an aromatic group, including, for example, a phenyl group. In some embodiments, each of C^1 and C^2 may be substituted, wherein one, two, or three carbon atoms in C^1 or C^2 may be optionally replaced with S, SO, O, F, N, NH, among other atoms, in a

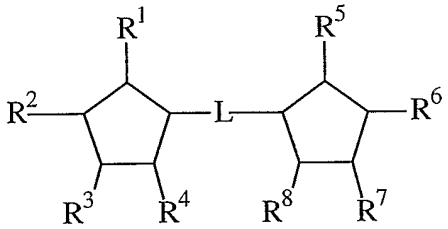
chemically stable arrangement. As used herein, a "chemically stable arrangement" refers to a compound structure that renders the compound sufficiently stable to allow manufacture and administration by methods known in the art. Typically, such compounds are stable at a temperature of 40 degrees Celsius or less, in the absence of moisture or other chemically reactive condition, for at least a week.

In some embodiments, either of C^1 or C^2 or both C^1 and C^2 may be a five membered ring, as shown below:



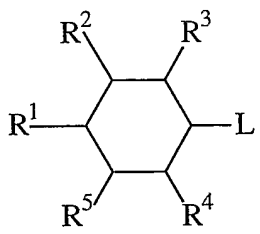
wherein each of R^1, R^2, R^3, and R^4 are independently selected from H, F, NO_2, an alkyne, an alkene, a ketone, an aldehyde, an ester or an amine.

In some embodiments, a derivative of 1,3-Bis(4-nitrophenyl)urea may have the structure as shown below:



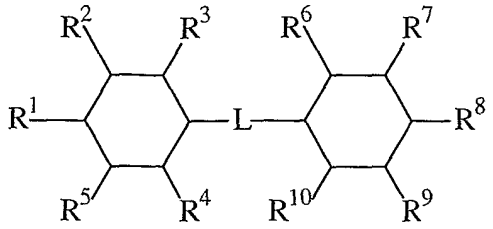
wherein each of R^1, R^2, R^3, R^4, R^5, R^6, R^7, and R^8 are independently selected from H, F, an alkyne, an alkene, a ketone, an aldehyde, an ester or an amine.

In some embodiments either of C^1 or C^2 or both C^1 and C^2 may be a six membered carbon ring, as shown below:



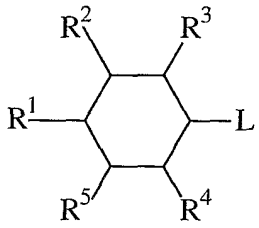
wherein each of R^1, R^2, R^3, R^4, and R^5 are independently selected from H, F, an alkyne, an alkene, a ketone, an aldehyde, an ester, or an amine.

In some embodiments, a derivative of 1,3-Bis(4-nitrophenyl)urea may have the structure as shown below:



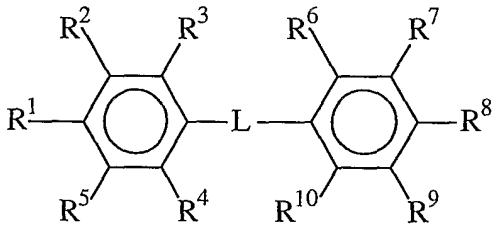
wherein each of R^1, R^2, R^3, R^4, R^5, R^6, R^7, R^8, R^9, and R^{10} are independently selected from H, F, an alkyne, an alkene, a ketone, an aldehyde, an ester, or an amine.

In some embodiments either of C^1 or C^2 or both C^1 and C^2 may be a phenyl group, as shown below:

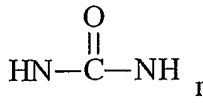


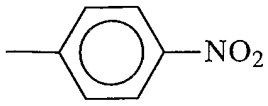
wherein each of R^1, R^2, R^3, R^4, R^5, R^6, R^7, R^8, R^9, and R^{10} are independently selected from H, F, an alkyne, an alkene, a ketone, an aldehyde, an ester, or an amine.

In some embodiments, a derivative of 1,3-Bis(4-nitrophenyl)urea may have the structure as shown below:

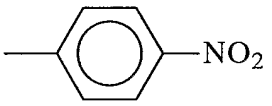


wherein R^1, R^2, R^3, R^4, R^5, R^6, R^7, R^8, R^9, and R^{10} are independently selected from H, F, an alkyne, an alkene, a ketone, an aldehyde, an ester or an amine; and

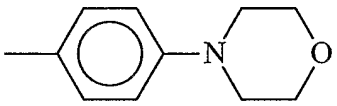
with the proviso that when L is a  moiety,

then R^1 and R^8 are not both a  moiety.

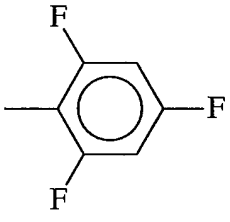
In some embodiments, any R^1, R^2, R^3, R^4, R^5, R^6, R^7, R^8, R^9, or R^{10} may be:



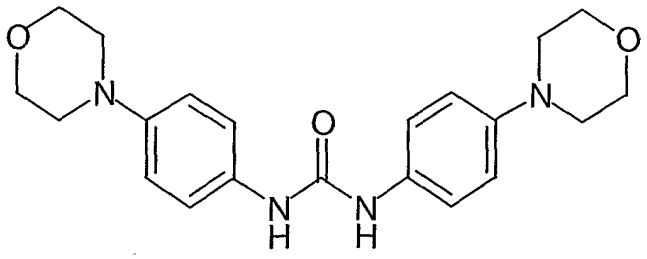
In some embodiments, any R^1, R^2, R^3, R^4, R^5, R^6, R^7, R^8, R^9, or R^{10} may be:



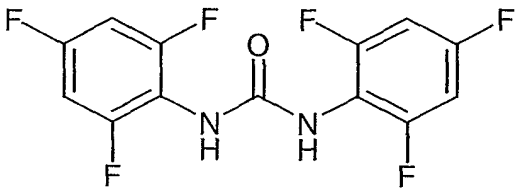
In some embodiments, any R^1, R^2, R^3, R^4, R^5, R^6, R^7, R^8, R^9, or R^{10} may be:



Derivatives of 1,3-Bis(4-nitrophenyl)urea include, for instance,



and



Derivatives of 1,3-Bis(4-nitrophenyl)urea include 1,3-Bis(4-nitrophenyl)urea derivatives in which different types of ureas have been incorporated, including symmetrical or asymmetrical (also referred to herein as "unsymmetrical") ureas derivatives.

Symmetrical urea derivatives include symmetrical bisaromatic ureas, possessing groups with properties similar to nitro group (NO_2). These ureas may have benzene rings substituted with NO_2 in the *ortho* and *meta* positions as well as sulfate (SO_3^-), acetate (OAc) and nitrile (CN) in the *ortho*, *meta*, and *para* positions. Both OAc and SO_3^- are electron-withdrawing groups like NO_2 and consequently provided aromatic rings with electron densities similar to the lead compound, 1,3-Bis(4-nitrophenyl)urea. Other 1,3-Bis(4-nitrophenyl)urea derivatives may contain symmetrical heteroaromatic groups such as imidazole, pyridine, thiazole and triazoles ureas. These members will possess a shape and Π-cloud similar to simpler aromatics while presenting additional hydrogen bonding options for further derivatization.

Unsymmetrical urea derivatives (also referred to herein as asymmetrical urea derivatives) of 1,3-Bis(4-nitrophenyl) include ureas with two different aromatic groups and ureas with one aromatic group and one aliphatic group. The unsymmetrical bisaromatic ureas may contain combinations of the aromatic groups discussed above. The aromatic/aliphatic ureas may use the aromatic groups with both acyclic and cyclic aliphatic groups. Such ureas may be synthesized from commercially available amines, of which more than 200

amines are available from Aldrich alone. Symmetrical urea derivatives of 1,3-Bis(4-nitrophenyl)urea include those obtained by the addition of two equivalents of an amine to a solution of phosgene or carbonyldiimidazole. Such unsymmetrical ureas are accessible by stepwise addition of one amine to carbonyl diamidazole followed by addition of the second amine. 1,3-Bis(4-nitrophenyl)urea derivatives include symmetrical ureas derivatives synthesized by reaction of aniline derivates with carbonyldiimidazole (Zhang et al., J. Org. Chem. 62:6420-6423, 1997).

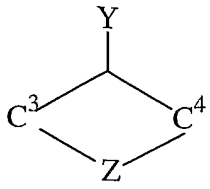
1,3-Bis(4-nitrophenyl)urea derivatives also include unsymmetrical ureas, synthesized, for example, on a solid support (Zheng and Combs, J. Comb Chem. 4:38-42, 2002). This includes, for example, when a support has been prepared with one half of the urea attached to the polymer as the initial step to perform the solid phase synthesis of ureas. This carbonyl diamidazole adduct allows twenty different amines to be added to different batches of the support to provide different unsymmetrical ureas. The parent thioxanthone moiety can be synthesized by reaction of 3-bromothiophenol with 2-fluorocyanobenzene followed by treatment with sulfuric acid to hydrolyze the nitrile and perform the Friedel-Crafts acylation in one step (Rewcastle et al., J. Med. Chem. 34, 491-496, 1991; Kristensen et al., J. Org. Chem. 68, 4091-4092, 2003). This can be followed by oxidation. Derivatives can be synthesized by conversion of the bromide to a variety of different groups using palladium catalyzed coupling reactions (Brase et al., Tetrahedron 59, 885-939, 2003).

1,3-Bis(4-nitrophenyl)urea derivatives include symmetrical and unsymmetrical functional group derivatives, where each position, or a combination of positions, on the aromatic ring could be substituted with a new functional group or chemical moiety (for example, methyl, hydroxyl, amine, or aldehyde. Derivatives include substitutions of a carbon position, or a combination of carbon positions, in the aromatic ring with different chemical entities that could include nitrogen, oxygen, and sulfur, among other atoms.

3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate is commercially available, for example, from Sigma-Aldrich.

In some embodiments, a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one 10,10-dioxide monohydrate includes a compound represented by

structural Formula II, or a pharmaceutically acceptable salt, solvate, ester, or prodrug thereof. Formula II is as provided below:



Formula II

wherein C^3 and C^4 are independently selected organic groups;

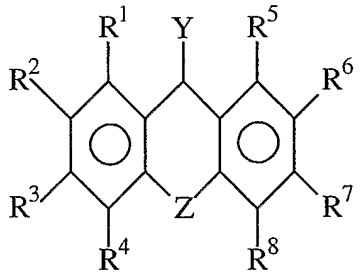
wherein Z is, for example, SO_2, O, C, a nitrogen, such as for example, NH, C; and

wherein Y is, for example, O, H, S, or an imine.

In some embodiments of Formula II, Z is SO_2.

In some embodiments of Formula II, Y is O.

In some embodiments, a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one 10,10-dioxide monohydrate includes a compound represented by structural Formula III, or a pharmaceutically acceptable salt, solvate, ester, or prodrug thereof. Formula III is as provided below:



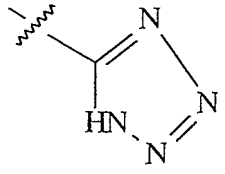
Formula III

wherein Z is, for example, SO_2, O, or a nitrogen, such as for example, NH;

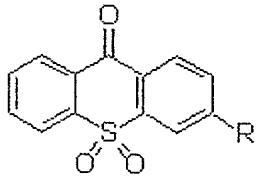
wherein Y is, for example, O, H, S, or an imine; and

wherein each of R1 to R8 is independently selected from H, a C1-C20 organic group, including for example, an aromatic group or a heteroaromatic group, an amide, a boronic acid, an alkyl, a vinyl, a carboxylic acid derivative, or an alkynal;

with the proviso that when Z is SO_2 and Y is O, then R7 can not be a


moiety.

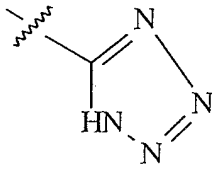
In some embodiments, a derivative of 3-(1H-tetrazol-5-yl)-9H-thioxanthen-9-one 10,10-dioxide monohydrate has the structure represented by structural Formula IV, below:



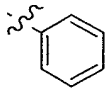
Formula IV

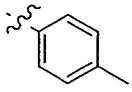
wherein R is

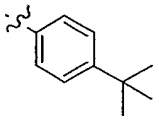
with the proviso that R is not a

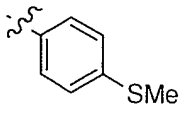

moiety.

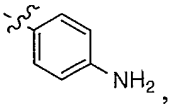
In some embodiments of Formula IV, R may be

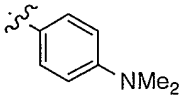
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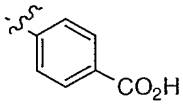
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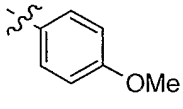
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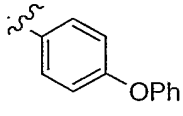
,

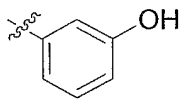

NH2
,

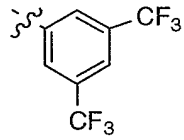

NMe2
,

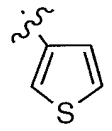

CO2H
,

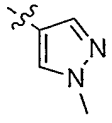

OMe
,

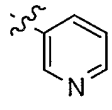

OPh
,

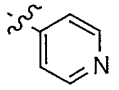

OH
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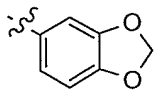

CF3
CF3
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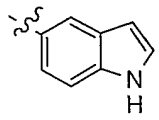

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Derivatives of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate include derivatives obtained by the addition of an R group to one or more different positions around the aromatic ring of the parent 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate compound. Derivatives include, for example, the addition of alkynes, alkenes, ketones, aldehydes, esters and/or amines to different positions around the parent aromotic ring.

The R group added can be provided by any of the many commercially available carboxy amides or boronic acids.

Derivatives may also include functional group derivatives, where each position, or combination of positions, on the aromatic rings could be substituted with a new functional group or chemical moiety (for example, methyl, hydroxyl, amine, or aldehyde). In addition, each carbon position, or a combination of carbon positions, in the aromatic rings could be substituted with different chemical entities that could include nitrogen, oxygen, and sulfur, among other atoms.

The addition of R groups may be via the addition of an intermediate bromide or chlorine to different positions around the parent aromatic rings. Bromothiophenols are commercially available. A wide variety of additional derivatives of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate may be obtained with palladium catalyzed couplings. Additional derivatives of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate may be obtained through the use of known reactions, including, for example, Suzuki and Sonogashira couplings (Kotha et al., Tetrahedron

58:9633-9695, 2002; Herrmann, The Suzuki Cross-Coupling Applied Homogeneous Catalysis with Organometallic Compounds (2nd Edition) 1:591-598, 2002; Herrmann et al., J. of Organometallic Chem. 687:229-248, 2003; Tykwinski, Angew. Chemie, Int. Ed. 42:1566-1568, 2003) as the Stille and Heck reactions (Duncton and Pattenden, The Intramolecular Stille Reaction Journal of the Chemical Society, Perkin Transactions 1: Organic and Bio-Organic Chemistry, 1235-1246, 1999; de Meijere et al., J. of Organometallic Chem. 653:129-140, 2002; Link, The Intramolecular Heck Reaction Organic Reactions (New York) 60:157-534, 2002) and Hartwig-Buchwald chemistry (Maes et al., Journal of Heterocyclic Chemistry 39:535-543, 2002; Yong and Nolan, Chemtracts 16:205-227, 2003), to add alkynes, alkenes, ketones, aldehydes, esters, or amines to a bromide.

Further, the synthetic chemistry as outlined by Batchelor et al. (AU 31113/77), Hodson et al. (U.S. Patent No. 4, 103,015), or Hodson et al., (UK-1447032) may be utilized to accomplish substitutions at varying position in the parent aromatic rings.

Derivatives of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate include derivatives with a central ring structure that has six or more carbons. Derivatives include, for example, compounds with a central ring that include six carbons, seven carbons, eight carbons, nine carbons, ten carbons, twelve carbons, or more. For example, a seven member carbon ring may be synthesized as detailed by Rokach et al. (U.S. Patent No. 4,536,507).

An agent of the present invention may demonstrate one or more of the following activities: inhibit or block the replication of a flavivirus in cell culture, inhibit or block the replication of a dengue virus in cell culture, inhibit or block the *in vivo* replication of a flavivirus, inhibit or block the *in vivo* replication of a dengue virus, inhibit or block a flavivirus protease activity, inhibit or block flaviviral NS3 protease activity, inhibit or block dengue virus NS3 serine protease activity, reduce the titer of infectious virus produced in cells challenged or infected with a flavivirus, and/or reduce the titer of infectious virus produced in cells challenged or infected with a dengue virus.

The activity of an agent may be assayed by any of many art known methods, including any of those described herein. Any of many cell-free or

enzyme based protease activity assays may be used. For example, for HCV methods that may be used include those described by Steinkuhler et al., J Virol. 70:6694-6700, 1996; Sudo et al., Antiviral Res. 32:9-18, 1996; Takeshita et al., Anal Biochem 247:242-246, 1997; Liu et al., Anal Biochem 267:331-335, 1999; Taliani et al., Anal Biochem 240:60-67, 1996, Lemon et al. (WO 05/053516), and Lemon et al. (U.S. Paten No. 6,921,634).

A cell-based screening method may be used. See, for example, Lee et al., Assay Drug Dev Technol. 3(4):385-392, 2005 for an example of a high-throughput cell-based screening method for Hepatitis C virus NS3/4A protease inhibitors)

Activity may be assayed in one of the various animal models that are available as models for human disease. For example, West Nile virus is lethal to mice within 6-7 days using either intracerebral or intraperitoneal routes of inoculation with clear evidence of encephalitis. Moreover, the WNV New York 1999 strain has an LD50 of approximately 1 pfu by intraperitoneal injection. Powassan virus type strain LB, a BSL-3 model for CEE virus, is lethal to mice 6-8 days post-infection with an LD50 of approximately 12 pfu by the intraperitoneal route.

Activity may be assayed in an animal model for dengue virus infection, including, for example models using a laboratory-adapted DENV strains to cause encephalitis following intracranial inoculation of suckling mice (Sabin, Am. J. Trop. Med. Hyg. 1:30-50, 1952). Encephalitis and death have been used as endpoints in some studies for evaluating vaccine efficacy in mice, and antiviral drugs may also show efficacy in this model (Koff et al., Antimicrob Agents Chemother. 24:134-6, 1983). Immunodeficient mice models for evaluation of DENV virulence/pathogenesis may be used. See, for example, Lin et al., K562 cells. J Virol 72, 9729-37, 1998; An et al., Virology 263, 70-7, 1999; and Johnson and Roehrig, J Virol 73, 783-6, 1999.

The agents of the present invention may be effective against one or more of the four dengue virus serotypes: dengue-1, dengue-2, dengue-3, and dengue-4. These viruses form an antigenically distinct subgroup within the flavivirus family (Calisher et al., J Gen Virol 70:37-43, 1989). Dengue viruses are the most common cause of arboviral disease in the world. They are found virtually throughout the tropics and cause an estimated 1-2 million clinical illnesses

annually, including 250,000-500,000 cases of dengue haemorrhagic fever, a severe manifestation of dengue, and about 254,000 deaths. More than two fifths of the world's population (2.5 billion) live in areas potentially at risk for dengue (reviewed by Gibbons and Vaughn in "Dengue: an escalating problem," Gibbons et al., BMJ 324(7353):1563-6, 2002).

Infection with any one of four dengue viruses produces a spectrum of clinical illness ranging from a mild undifferentiated febrile illness to dengue fever (DF) to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), both potentially life-threatening diseases. While no specific preventive or therapeutic agents exist for dengue, morbidity and mortality can be reduced by early hospitalization and careful supportive care (Libraty et al., J Infect Dis. 186(8):1165-8, 2002).

No cures or effective therapeutics exist to combat DENV infection. Treatment typically involves supportive care, including prolonged hospitalization and intensive care resources as the disease progresses to DHF and DHSS (dengue hemorrhagic shock syndrome). Vaccines have been investigated as possible tools to protect against dengue virus; however epidemiological evidence indicates that immunity to one serotype of DENV increases the chance of a more severe disease upon infection with a second serotype by about ten-fold (Kurane and Ennis, Immunopathogenesis of dengue virus infections. Dengue and Dengue Hemorrhagic Fever. D. J. Gubler and G. Kuno. Oxon, UK, CABI Publishing, 273-290, 1997). Although in vitro data support the hypothesis that pre-existing, cross-reactive antibodies enhance viral replication during a second encounter with DENV (a process know as antibody-dependent enhancement of infection or ADE), the precise involvement of pre-existing immunity to DENV infection in immunopathogenesis of dengue remains unclear. Concern that ADE will occur among vaccines, making vaccinated individuals more susceptible to severe disease, has proven a major obstacle in the testing of dengue vaccine candidates, slowing the development of much-needed dengue vaccines. Moreover, vaccines provide little protection to an unvaccinated population exposed to deliberate DENV release. These concerns increase the potential value of dengue antivirals as alternatives to vaccination. Both ribavirin and inosine monophosphate dehydrogenase (IMPDH) inhibitors (for example, mycophenolic acid (also referred to herein as

"MPA")) have been studied as potential flavivirus antivirals (Leyssen et al., Clinical Microbiology Reviews 13:67-82, 2000; Diamond et al., Virology 304:211-21, 2002). While effective at suppressing viral replication in cell culture, sub-toxic concentrations of these compounds showed little sustained virus clearance in animals. The exact mechanisms of action of ribavirin and MPA are unknown; however neither compound appears to specifically inhibit viral proteins (Benarroch et al., J Biol Chem. 279:35638-43, 2004).

Following infection of its human host by the bite of an infected mosquito, dengue virus undergoes local replication, infecting several cell types, including dendritic cells. This initial infection is followed by systemic infection of monocytes, resulting in a high-level viremia that can last for several days. Infection of macrophages and monocytes in lymphoid organs and the circulation results in the malaise, fever, and rash that characterize the clinical syndrome "dengue fever" (McBride and Bielefeldt-Ohmann, Microbes Infect 2:1041-50, 2000). In a subset of infections, a second peak of viremia is observed approximately one week after initial exposure; this peak can be associated with the more severe forms of the disease, including DHF, which has been associated with a strong T cell response to dengue virus infection (Rothman, Adv Virus Res 60:397-419, 2003). Although there are some indications that severe forms of dengue infections can result in neurological symptoms, encephalitis is not considered to be an important outcome of DENV infection. Several years ago, Vaughn et al. observed that peak viremias detected in patients were associated with increased clinical severity (Vaughn et al., Journal of Infectious Diseases, 181:2-9, 2000), with significantly different clinical outcomes noted for patients displaying modest (approximately 100- fold) differences in peak viremias. More recently, Libraty et al. demonstrated that high levels of NS1 protein antigenemia (which correlates with viremia) were associated with more severe forms of dengue disease (Libraty et al., J Infect Dis. 186:1165-8, 2002). These data, taken together with the fact that subclinical dengue infections have been noted for decades, strongly indicate that antivirals that moderately reduce or attenuate viral load could help ameliorate the primary phase of the infection (including the symptoms of dengue fever) and prevent the later stage, more severe hemorrhagic and shock manifestations that result from dengue viral

infection. Thus, dengue represents an ideal disease for post-exposure (and/or post-presentation) treatment with antiviral drugs.

Further evidence that dengue disease could be managed by chemotherapeutics comes from research indicating that infection of non-human primates results in a transient (usually less than four days) viremia that is at least two orders of magnitude lower than that observed in man, and that these animals do not display any signs of disease (Halstead et al., J Infect Dis. 128:7-14, 1973; and Halstead et al., J Infect Dis. 128:15-22, 1973).

Although dengue causes the greatest number of cases of human disease of any flavivirus, Japanese encephalitis virus and yellow fever are also important diseases that affect hundreds of thousands of people each year. In addition, approximately 10,000 confirmed cases of West Nile virus infection occurred in the United States in 2003. Furthermore, there is evidence that yellow fever virus and the tick-borne encephalitis viruses, which can display case-fatality rates of up to 50%, have been developed for use as bioweapons. Thus, broad-spectrum antivirals that inhibit infection by dengue and other flaviviruses could combat these bioterrorist agents and significantly improve Homeland Defense and global public health.

The recent establishment of West Nile virus in North America demonstrates the long-term, damaging consequences that can arise from introduction of a flavivirus disease agent into the US. Although the broad vertebrate and invertebrate host specificity of West Nile virus may have predisposed North America to this incursion, there are multiple mosquito vectors of Japanese encephalitis virus, yellow fever virus, and dengue virus in the US, indicating that these diseases could also become established, and yellow fever was established in many US cities in the late 1800s. Furthermore, the fear and public anxiety that West Nile virus outbreaks have generated, the speed of its spread across the US, and the association of its spread with organ donation and transfusion demonstrate that flaviviruses can terrorize a civilian population even without intentional malicious introduction.

West Nile virus is a mosquito-borne flavivirus with a rapidly expanding global distribution. Infection causes severe neurological disease and fatalities in both human and animal hosts. West Nile virus is transmitted via mosquitoes from avian reservoir hosts to vertebrate dead end hosts that include humans and

horses. While endemic in humans in parts of Africa, Europe and the Middle East, recent outbreaks in Israel (1998), Romania (1996), and the United States (1999), have been associated with serious neurological pathology and fatal infections. During the last five years, West Nile virus has spread rapidly throughout the USA, Canada and Mexico, as well as appearing recently in the United Kingdom. This rapid global spread, through developed countries, has prompted widespread implementation of prevention strategies. No vaccines or therapeutic treatments for West Nile virus infections are yet available. The West Nile viral protease (NS2B-NS3) is essential for post-translational processing in host-infected cells of a viral polypeptide precursor into structural and functional viral proteins and its inhibition presents a potential treatment for viral infections. See Nall et al., J Biol Chem. 279:48535-42, 2004.

Approximately 170,000,000 people worldwide and 4,000,000 in the United States are infected with Hepatitis C virus (HCV). The virus is transmitted primarily by blood and blood products. The majority of infected individuals have either received blood transfusions prior to 1990 (when screening of the blood supply for Hepatitis C virus was implemented) or have used intravenous drugs. Sexual transmission between monogamous couples is rare but HCV infection is more common in sexually promiscuous individuals. Perinatal transmission from mother to fetus or infant is also relatively low but possible (less than 10%). Many individuals infected with HCV have no obvious risk factors. Most of these persons have probably been inadvertently exposed to contaminated blood or blood products.

About 85% of individuals acutely infected with HCV become chronically infected. Hence, HCV is a major cause of chronic (lasting longer than six months) hepatitis. Once chronically infected, the virus is almost never cleared without treatment. In rare cases, HCV infection causes clinically acute disease and even liver failure, however, most instances of acute infection are clinically undetectable.

The natural history of chronic HCV infection can vary dramatically between individuals. Some will have clinically insignificant or minimal liver disease and never develop complications. Others will have clinically apparent chronic hepatitis. Of these, some go on to develop cirrhosis, however, the exact percentages is not known. About 20% of individuals with hepatitis C who do

develop cirrhosis will develop end-stage liver disease. Cirrhosis caused by hepatitis C is presently the leading indication for orthotopic liver transplantation in the United States. Individuals with cirrhosis from hepatitis C are also at an increased risk of developing hepatocellular carcinoma (primary liver cancer). A major problem in discussing prognosis in patients with chronic hepatitis C is that it is difficult to predict who will have a relatively benign course and who will go on to develop cirrhosis or cancer.

As 1,3-Bis(4-nitrophenyl)urea and 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate target different sites of the dengue 2 NS3 serine protease, the present invention includes bivalent compounds that include as one component, 1,3-Bis(4-nitrophenyl)urea or a 1,3-Bis(4-nitrophenyl)urea derivative, and as a second component, 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate derivative.

The present invention further includes bivalent compounds, wherein one component of the bivalent compound is 1,3-Bis(4-nitrophenyl)urea, a 1,3-Bis(4-nitrophenyl)urea derivative, 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate derivative, and a second component of the bivalent compound is an additional antiviral agent. Such an additional antiviral agent can be any of the various known antiviral agents.

The present invention includes methods of treating and/or preventing flaviviral infections. An agent of the present invention may be administered to a subject for the treatment of a flavivirus infection. An agent of the present invention may be administered to a subject prior to and/or after exposure to a flavivirus. An agent of the present invention may be administered to a subject prior to and/or after infection with a flavivirus. An agent may be administered in an amount effective to inhibit replication of the flavivirus. Agents of the present invention may be used as antiviral agents to reduce the replication and/or production of a flavivirus, such as dengue, in an infected individual. Inhibition of the replication of a flavivirus may be determined, for example, by methods including, but not limited to, methods described in the examples herein. An agent of the present invention may be administered in an amount effective to inhibit protease activity of a flavivirus protease, including, but not

limited to, protease activity of the NS3 protease of dengue virus. Inhibition of the protease activity of a flavivirus protease may be determined by methods including, but not limited to, those described in more detail in the examples herein, or by Leung et al., (J. Biol. Chem. 276:45762-45771, 2001).

An agent of the present agent may serve as a broad spectrum antiviral, effective for the treatment or prophylaxis of one or more flaviviral infections.

As used herein, a "subject" or an "individual" is an organism, including, for example, a mammal. A mammal may include, for example, a rat, mouse, a primate, a domestic pet (such as, but not limited to, a dog or a cat), livestock (such as, but not limited to, a cow, a horse, and a pig), or a human. By the term "effective amount" of an agent as provided herein is meant a nontoxic but sufficient amount of the agent or composition to provide the desired effect. The exact amount required will vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of the condition being treated, and the particular agent and its mode of administration, and the like. Thus, it is not possible to specify an exact "effective amount." However, an appropriate effective amount may be determined by one of ordinary skill in the art using only routine experimentation. Therapeutically effective concentrations and amounts may be determined for each application herein empirically by testing the compounds in known in vitro and in vivo systems, such as those described herein; dosages for humans or other animals may then be extrapolated therefrom.

An agent of the present invention may be administered to a subject to prevent the infection of a subject with a flavivirus. Agents of the present invention may be taken as a prophylactic to prevent the development of a flavivirus infection. This may be particularly beneficial for travelers or visitors to locations were flavivirus infections are endemic or regions experiencing an outbreak or epidemic with a flavivirus.

An agent of the present invention may be administered to a subject to reduce the severity of the symptoms associated with a flavivirus infection. The agent may be administered to a subject prior to and/or after exposure to or infection with a flavivirus. The symptoms of a flavivirus infection that may be reduced in severity by the administration of an agent of the present invention may include one or more of the following: high fever, severe headache, nausea,

vomiting, flushed facies, sore throat, cough, cutaneous hyperaesthesia, taste aberrations, headache, retro-orbital pain, myalgia, arthralgia, encephalitis, neurological manifestations, haemorrhagic manifestations, rash, including, but no limited to maculopapular rash and macular rash, severe haemorrhage, thrombocytopenia, consumptive coagulopathy, vascular leak syndrome, clinical shock, leucopenia, haemorrhagic manifestations (shown, for example, by positive tourniquet test, petechiae, ecchymoses or purpura, or bleeding from mucosa, gastrointestinal tract, injection sites, or other locations), platelet count of less than 100,000/mm^3, plasma leakage, vascular permeability, pleural effusion, ascites, hypoproteinaemia pulse pressure of less than 20 mm Hg, and/or hypotension (defined as systolic pressure less than 80 mm Hg for those less than five years of age or less than 90 mm Hg for those over five years of age).

The clinical features of dengue vary with the age of the patient and can be classified into five presentations; non-specific febrile illness, classic dengue, dengue haemorrhagic fever, dengue haemorrhagic fever with dengue shock syndrome, and other unusual syndromes such as encephalopathy and fulminant liver failure. A severe manifestation of dengue is dengue haemorrhagic fever (DHF), which is more common after a second infection with dengue virus. DHF is infection characterized by plasma leakage and is distinguished from dengue by the presence of increased vascular permeability, not by the presence of haemorrhage. For infection with a dengue virus, an agent of the present invention may be administered to prevent the development of and/or reducing the severity of the symptoms of dengue hemorrhagic fever and/or dengue shock syndrome.

The agents of the present invention may be administered at once, or may be divided into a number of smaller doses to be administered at intervals of time. It is understood that the precise dosage and duration of treatment is a function of the desired therapeutic outcome and may be determined empirically using known testing protocols or by extrapolation from in vivo or in vitro test data. It is to be noted that concentrations and dosage values may also vary with the severity of the condition to be treated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person

administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed compositions and methods. The agents of the present invention may be administered to the subject in combination with other therapeutic modalities. The agents of the present invention can be administered before, during or after the administration of the other therapies.

The agents of the present invention may be formulated in a composition. A composition may include one or more of 1,3-Bis(4-nitrophenyl)urea, a derivative of 1,3-Bis(4-nitrophenyl)urea, 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, and a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate.

In some aspects of the present invention, compositions including 1,3-Bis(4-nitrophenyl)urea or a derivative thereof do not include 2-hydroxy 4,6-dimethylpyrimidine. In some aspects, the present invention includes compositions that do not include nicarbazin, an equimolecular complex of 4,4'-dinitrocarbanilide with 2-hydroxy-4,6-dimethylpyrimidine. Nicarbazin has been used in the poultry industry, where it has been administered to birds to reduce the incidence of encephalopathy in young chickens (Bartov and Budowski, Poult Sci. 58(3):597-601, 1979), control the protozoal disease coccidiosis (Rogers et al., Science 222(4624):630-2, 1983) and to arrest egg production (Bar et al., Poult Sci. 82(4):543-50, 2003).

Agents of the present invention may be administered in a composition including a pharmaceutically acceptable carrier. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, that is, the material may be administered to an individual along with an agent of the present invention without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained.

Agents of the present invention may be formulated in a composition along with a "carrier." As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for

pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated.

Supplementary active ingredients can also be incorporated into the compositions of the present invention, including, but not limited to, antiviral, anti-fever and/or pain medications. Compositions of the present invention may include additional antiviral agents, such as, for example, ribovarin, AZT, and any of the various antiviral compound disclosed in U.S. Patent No. 6,914,054, U.S. Patent Application 2005/0215486, U.S. Paten Application 2005/0245458, Beaulieu et al., J Org Chem. 70(15):5869-79, 2005; Venkatraman et al., J Med Chem. 48(16):5088-91, 2005; and Goudreau et al., Expert Opin Investig Drugs 14(9):1129-44, 2005. In certain embodiments, the agents of the present invention may be contained within a time-released composition.

The present invention includes methods for identifying agents suitable for the treatment or prevention of a flavivirus infection. Such a method may include contacting cells with a flavivirus and an agent that is a derivative of 1,3-Bis(4-nitrophenyl)urea, wherein the production of a decreased flavivirus titer indicates the agent is suitable for the treatment or prevention of a flavivirus infection. Such a method may include contacting cells with a flavivirus and an agent that is a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, wherein the production of a decreased flavivirus titer indicates the agent is suitable for the treatment or prevention of a flavivirus infection. Such a method may include contacting a flavivirus protease with an agent that is a derivative of 1,3-Bis(4-nitrophenyl)urea, wherein an inhibition of the protease activity of the flavivirus protease indicates the agent is suitable for the treatment or prevention of a flavivirus infection. Such a method may include contacting a flavivirus protease with an agent that is a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, wherein an inhibition of the protease activity of the flavivirus protease indicates the agent is suitable for the treatment or prevention of a flavivirus infection. Methods for assaying for the inhibition of a flavivirus protease include, for example, the methods described in the examples herein.

The present invention includes methods for identifying agents suitable for the treatment or prevention of a dengue viral infection. Such a method may

include contacting the NS3 serine protease of a dengue virus with an agent that is a derivative of 1,3-Bis(4-nitrophenyl)urea, wherein the inhibition of the serine protease activity of the NS3 serine protease of a dengue virus indicates the agent is suitable for the treatment or prevention of a dengue virus infection. Such a method may include contacting the NS3 serine protease of a dengue virus with an agent that is a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, wherein the inhibition of the serine protease activity of the NS3 serine protease of a dengue virus indicates the agent is suitable for the treatment or prevention of a dengue virus infection. Methods for assaying for the inhibition of a dengue NS3 serine protease include, for example, the methods described in the examples herein and as described in Leung et al, J. Biol. Chem. 276:45762-45771, 2001.

The present invention includes combinatorial chemistry libraries (also referred to herein as "combinatorial libraries") that include at least one derivative of 1,3-Bis(4-nitrophenyl)urea and/or at least one derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate. Combinatorial chemistry libraries of the present invention may include a multiplicity of such derivatives. Such combinatorial chemistry libraries may also include 1,3-Bis(4-nitrophenyl)urea and/or 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate.

A combinatorial library of the present invention can be built around the lead antiviral 1,3-Bis(4-nitrophenyl)urea. A combinatorial library of the present invention can be built around the lead antiviral 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate.

In a combinatorial chemistry library a large number compounds can be synthesized and screened for various possible physiological or other activities. Combinatorial chemistry allows scientists to generate large numbers of unique molecules with a small number of chemical reactions. Rather than using the traditional approach of synthesizing novel compounds one at a time, compounds are synthesized by performing chemical reactions in stages, and reacting all of the molecules formed in a given stage with a given reactant. This process is iterated until the desired end products are produced. The diverse library of molecules thus formed may be used to screen for biological activity against a therapeutic target or for any other desirable property. Numerous strategies have

been devised for producing such combinatorial libraries. See, for example: Baum, "Combinatorial Approaches Provide Fresh Leads for Medicinal Chemistry, Chemical & Engineering News, Vol. 72, Feb. 7, 1994, pp. 20-26; Furka et al., "Cornucopia of Peptides by Synthesis," Abstr. 14th Int. Cong. of Biochem., Prague Czechoslovakia, 5:47, 1988; "More Peptides by Less Labour," Abstr. 10th Int. Sym. on Med. Chem., Budapest, Hungary p 288 (1988); Burbaum et al., "A Paradigm for Drug Discovery Employing Encoded Combinatorial Libraries," Proc. Natl. Acad. Sci. USA, 92:6027-6031, 1995; Baldwin, "Design, synthesis and use of binary encoded synthetic chemical libraries," Mol Divers., Vol. 2(1-2):81-8, 1996; Baldwin et al., "Synthesis of a Small Molecule Combinatorial Library Encoded with Molecular Tags," Journal of the American Chemical Society, 117(20):5588-5589, 1995; Nestler et al., "A General Method for Molecular Tagging of Encoded Combinatorial Chemistry Libraries," The Journal of Organic Chemistry, 59(17):4723-4724, 1994; Borchardt et al., "Synthetic Receptor Binding Elucidated with an Encoded Combinatorial Library," Journal of the American Chemical Society, 116:373-374, 1994; Ohlmeyer et al., "Complex Synthetic Chemical Libraries Indexed with Molecular Tags," Proc. Natl. Acad. Sci. USA, 90:10922-10926, 1993; "The Promise of Combinatorial Chemistry," Windhover's In Vivo The Business & Medicine Report, Vol. 12, No. 5, May, 1994, pp. 23-31; WO 94/08051; and U.S. Patent Nos. 5,663,046 and 6,377,895. Definitions for the various terminology used in association with combinatorial libraries and combinatorial chemistry may be as found in the "Glossary of Terms Used in Combinatorial Chemistry (Technical Report)" (Clean et al., Pure Appl. Chem., 71(12):2349-2365, 1999).

Unless otherwise specified, "a," "an," "the," and "at least one" are used interchangeably and mean one or more than one.

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

EXAMPLES

Example 1

Identification of lead antiviral compounds

No antivirals or vaccines exist to combat dengue virus (DENV) infection. This is a serious failing since dengue virus is responsible for approximately 1.5 million clinical cases annually, causes severe hemorrhagic fever (DHF) in large numbers of infected individuals, and is a recognized Category A bioterrorist agent. To address the lack of therapeutics for this high priority bioterorrism agent, this example summarizes the identification of two low molecular weight compounds with activity in cell culture against dengue and West Nile (WN) replicons. Sub-toxic concentrations of the lead antivirals are active in cell culture against dengue 2 virus (DEN2V) and WN replicons. Based on the chemical structures of these lead antivirals, combinatorial chemical libraries representing second-generation antivirals will be generated. To select antivirals with broad-spectrum activity, these libraries will be screened in cell culture for efficacy against the four dengue virus serotypes, West Nile virus, and yellow fever virus. Since poor drug-like properties, including, for example, bioavailability, pharmacokinetics, metabolism, or toxicity, are the primary reason drug leads fail to progress beyond preclinical trials, this example stresses simultaneously optimizing drug properties and compound potency. Promising antivirals will be evaluated in vitro for solubility and cytochrome P450 inhibition, and evaluated in vivo for toxicity, pharmacokinetic properties, and bioavailability. Qualitative structure-activity analysis of compound potency and drug-like properties will be used to refine subsequent combinatorial libraries to optimize their antiviral drug potential and optimized antivirals will be readied for final investigation new drug (IND) enabling studies and Phase I-III clinical trials. These trials will yield well-tolerated drugs that can be administered to reduce the severity and progression of dengue infection, or used for post-exposure prophylaxis following a bioterrorist incident. It is expected that the present example will generate orthogonal classes of optimized antivirals, as the two lead antiviral compounds

have different chemical structures and are predicted to target independent protease sites

Computer-Based Antiviral Discovery. Computer screening of virtual chemical libraries binding to DEN2V NS3 protease identified commercially available small molecules (1,3-bis (4-nitrophenyl) urea and 3-(1H-tetrazol-5-yl)-9H-thio- xanthen-9-one 10,10-dioxide monohydrate) as potential NS3 inhibitors. These compounds have confirmed antiviral activity in cell against DEN2V and WN replicons. Molecular modeling studies indicate that these antivirals interact with independent NS3 protease sites. These antivirals will serve as templates for synthetic combinatorial chemical libraries from which new therapeutics against Category A-C flaviviruses (for example, DENV, WNV, and YFV) and widespread pathogenic flaviviruses (for example, HCV) can be developed.

The recently developed EUDOC program (Pang et al., J Comput Chem. 22:1750-1771, 2001; Pang et al., FEBS Letters 502:93-7, 2001; and Dooley et al., Bioorg. & Med. Chem. Lett., in press, 2005) was used to suggest small molecules capable of docking to DEN2V NS3 protease. EUDOC is a computer program for the identification of drug interaction sites in macromolecules and drug leads from chemical databases. This program correctly reproduced observed protein-ligand interactions for a large set of test cases, and identified an inhibitors of human adenovirus cysteine protease and human severe acute respiratory syndrome-associated coronavirus (Pang et al., J Comput Chem. 22:1750-1771, 2001; Pang et al., FEBS Letters 502:93-7, 2001; and Dooley et al., Bioorg. & Med. Chem. Lett., in press, 2005). EUDOC uses a lock and key method of screening by incrementally moving a possible ligand in all three dimensions and rotations through a user defined area using an Amber force field for "energy" scoring. This approach allows for the rapid and effective searching of large compound databases. The two DEN2V NS3 protease structures were used as target molecules for virtual screening (Murthy et al., J Mol Biol. 301:759-67, 2000; Murthy et al., J Biol Chem. 274:5573-80, 1999). Although neither structure contained the NS2B co-factor required for protease activity, these structures were models of apo- and inhibitor- bound conformations. The spatially distinct catalytic site and the P1 pocket of both protease structures were targeted for the virtual screening (Fig. 3). There were

few conformational differences between the backbone atoms of apo protease and the protease complexed to the Bowman-Birk inhibitor. However, significant differences in the active site and P1 pocket side chain conformations were observed between the two structures.

A virtual library of approximately two million small molecule structures was screened. To increase the likelihood of selecting small molecules active in cell culture, the virtual library was filtered to remove compounds containing formal charges. To obviate the need for a labor-intensive synthetic chemistry program, the library was additionally filtered to retain only compounds available from reputable chemical suppliers. Virtual docking computations were performed using supercomputer resources. Potential inhibitor-protease complexes were selected for the apo and inhibitor-bound protease, with each complex having predicted interaction energies of approximately -58 kilocalories/mole (kcal/mol) and -34 kcal/mol for the catalytic and P1 binding sites, respectively. The chemical structures of the lower energy complexes obtained at each site were examined, and a small set of compounds that showed no obvious detrimental reactivity, toxicity or solubility properties were purchased for cell culture toxicity and dengue antiviral activity assays.

Cell culture toxicity of lead compounds. The approximate toxicity of each purchased EUDOC-suggested compound was determined by dilution series in cell culture media with a final solution containing 1 percent (%) DMSO. LLC-MK2 (rhesus monkey kidney epithelial) cells were incubated 24 hours with each compound and cells examined visually for cytotoxic effects (cpe). This crude measure of cpe dosage was used to establish concentration ranges for MTT cell proliferation assays necessary to accurately and quantitatively measure cpe with high sensitivity. Briefly, 96-well plates were seeded with LLC-MK2 cells and treated with dilution series of compounds. Cells were incubated with compounds for fixed time (typically 24-72 hours), media removed, and cells treated with MTT for approximately three hours. The MTT substrate was then removed, the blue aqueous-insoluble product suspended in isopropyl alcohol, and samples read at 562 nanometers (nm) using an ELISA plate reader. Cytotoxic effect (cpe) was normalized against control wells treated with media and 1% DMSO. Assay conditions were optimized to establish a linear relationship between cell number and signal produced.

Maximum tolerated dose (maximum concentration with no apparent cpe) and, where possible, cytotoxic concentration (CC50) were determined for each tested compound. MTT assays (Fig. 4) included ribavirin and myophenoic acid (MPA), and were highly reproducible (standard deviations approximately 7%). Maximum tolerated doses measured by MTT assay were consistent with those estimated from visual inspection of cpe.

Two EUDOC-suggested compounds reduced DEN2V production in cell culture. To maximize the likelihood of identifying EUDOC-suggested compounds with modest antiviral activity in cell culture, LLC-MK2 cells were treated overnight at approximately one tenth the maximum tolerated dose estimated for each compound, and then challenged with DEN2V (strain 16681) at low multiplicity of infection (also referred to herein as "MOI"). Media and compound were replaced every 24 hours. Cells and media were collected daily, and tested for DEN2V replication using slot-blot assays to measure viral proteins in the media and immuno-foci staining plaque assays to measure infectious particles released to the media. All assays consistently concluded that EUDOC-suggested compounds ARDP0011 (1,3-Bis(4-nitrophenyl)urea) and ARDP0012 (3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one 10,10-dioxide mono-hydrate) reduced DEN2V replication in cell culture.

Slot-blot assay. Aliquots of post-challenge media were treated with 10% sodium dodecyl sulfate (SDS), transferred to nitro-cellulose membrane using a standard slot blot apparatus (BioRad), and the membrane probed with mouse anti-DEN2V primary antibody. Antigen levels were measured by standard horseradish peroxidase assays. Reproducible slot-blot experiments showed EUDOC-recommended compounds ARDP001 and ARDP0012 reduced DEN2V protein accumulation in the media relative to control cells (Fig. 5). Cells were incubated with 3 uM ARDP0005, 3 uM ARDP0006, 3 uM ARDP0011, and 30 uM ARDP0012. No cpe was observed during the course of this experiment. Compounds ARDP0011 and ARDP0012 clearly reduced viral antigen levels (relative to infected control cells) during days one to three post-infection. At four to seven days post-infection, no consistent decrease in viral antigen was observed with these compounds. Slot-blot assays proved effective in identifying ARDP0011 and ARDP0012 for subsequent studies. These assays can be labor-intensive, generate abundant contaminated wastes, and produce

uneven background levels over the membrane. Thus, a high-throughput ELISA assay was developed and utilized for additional studies to quantitate DENV proteins within infected cells.

Immuno-foci staining plaque assay. The quantity of infectious particles produced is one of the most important variables for predicting pathogenesis. The titer of infectious DENV in cell media was quantitated as plaque forming units (pfu) per milliliter (ml) of cell culture. An immunohistochemical (immuno-foci) staining method measured titers, since standard plaque methods are notoriously difficult, unreliable, and irreproducible for DENV due to its very low cpe in cell culture. This immuno- foci assay followed standard plaque methods of infecting confluent monolayers of cells with serial dilutions of sample, then covering the cells with tragacanth gum approximately one hour after infected media was applied to the monolayer. The gum was removed after three days, cells fixed with cold methanol/acetone (1:1), and probed with the same set of antibodies used in the slot blot assay. The infected cells were visualized by staining with the VIP kit (Vector Labs). Since the immuno-foci staining assay is labor and time intensive, the antiviral effects of only a small subset of promising EUDOC-suggested compounds were examined. Ribavirin (a triazole carboxamide with broad antiviral activity in several cell lines) and 1% DMSO were included as positive and negative controls, respectively. DEN2V challenge experiments clearly showed compounds ARDP0011, ARDP0012, and ribavirin had significant antiviral activity in cell culture. A representative experiment is summarized in Table 1. During early growth phase (one to two days post infection), DEN2V production was reduced in cells incubated with ARDP0011, ARDP0012, and ribavirin, relative to cells incubated with 1% DMSO. At four days post infection, cells incubated with ribavirin produced approximately 40-fold less virus than cells incubated with 1% DMSO, while cells incubated with either ARDP0011 and ARDP0012 produced DEN2V at levels similar to control levels. This suggested that ARDP0011 and ARDP0012 were significant DEN2V inhibitors when virus loads were low, but were unable to inhibit virus production at high virus loads. Although the mechanism of antiviral activity observed for these compounds has not been conclusively demonstrated, the above observations are consistent with these compounds functioning as viral protease inhibitors with modest inhibition

constants. Thus, it is logical to expect that systematically improving the binding potential and drug-like properties of these lead antivirals will produce potent antivirals effective at nanamolar drug concentrations.

Table 1
Anti-DEN2V activity of selected compounds in LLC-MK2 cells. Mean DEN2V titers and standard deviations were recorded as pfu/ml. P-scores were calculated by a heteroscedastic t-test relative to virus titers in cells incubated with media and 1% DMSO.

Compound	Measured quantity	Days post infection 1	2	4
DMSO (1%)	mean titer	116	2980	2E+05
	std. dev.	48	1700	6.8+04
	Replicates	8	10	9
ARDP0011 (3 μM)	mean titer	47	1080	1.9E+05
	std. dev.	22	815	1E+05
	replicates	6	8	11
	p-score	2.6E-03	5E-03	0.74
ARDP0012 (30 μM)	mean titer	38	1420	2.5E+05
	std. dev.	21	670	1.4E+05
	Replicates	6	10	10
	p-score	1.1E-03	1.4E-02	0.39
Ribavirin (30 μM)	mean titer	2	42	4680
	std. dev.	4	34	3280
	replicates	6	6	11
	p-score	9E-05	1.9E-04	6.2E-06

Chemical properties of lead antivirals. Antivirals referenced by EUDOC database identifiers ARDP0011 and ARDP0012 are 1,3-Bis(4-nitrophenyl)urea and 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one 10,10-dioxide monohydrate, respectively (see Fig. 7). These low molecular weight compounds were readily obtained from Aldrich Chemical Co. Interestingly, nitrophenyl urea is one of two equimolar components of nicarbazin, which has been used for approximately fifty years as a starter feed supplement to prevent intestinal coccidiosis in broiler chickens. In 1998, the World Health Organization reviewed existing toxicological and pharmacokinetic (PK) data on nicarbazin, concluded that an Acceptable Daily Intake could be supported for nicarbazin given its long and widespread use in veterinary medicine with no evidence of toxic effects. (WHO International Programme on Chemical Safety, Toxicological Evaluation of Certain Veternairy Drug Residues in Food, WHO Food Additives Series 41, 50th meeting of the Joint FAO/WHO Expert,

Committee on Food Additives (JECFA), World Health Organization, Geneva, 1998).

NS3 protease binding sites are highly conserved among flaviviruses. The antivirals ARDP0011 and ARDP0012 were initially selected from an extensive computer-docking calculation that predicted these compounds would bind tightly to the DEN2V NS3 protease, with ARDP0011 binding to the P1 site and ARDP0012 binding to the protease catalytic site. Residues forming the predicted ARDP0011 and ARDP0012 binding sites are highly conserved among the major clades of the flavivirus family, indicating that these lead antivirals and derivatives may display antiviral activity for a broad spectrum of flaviviruses.

Lead activity in West Nile virus replicon assay. A non-infectious, sub-genomic form of the genome of West Nile virus (a replicon RNA) that is capable of replication in multiple cells types has been developed (Scholle et al., J Virol. 78:11605-14, 2004; Scholle et al., Virology, 342:77-87, 2005). A stable cell line containing a luciferase gene-bearing derivative of this replicon has been produced and is useful for antiviral screening. When tested with lead antiviral ARDP0011 and several other antiviral agents known to have activity against West Nile virus (Morrey et al., Antiviral Res 55:107-16, 2002), this method readily detected activity in a 96-well plate assay (Fig. 6). Thus, ARDP0011 has antiviral activity against two flavivirus clades. ARDP0012 will be tested in a similar fashion. This assay has demonstrated utility in identifying compounds that interfere directly with viral genome replication (such as polymerase or protease inhibitors), and should be particularly useful for this project. A similar replicon for Japanese encephalitis virus has been developed and a DENV replicon will be produced.

Cell-based dengue ELISA assay. A cell-based ELISA assay to detect viral proteins in infected cells is well-suited for high-throughput screening and can be performed more rapidly than virus yield assays. This assay also minimizes exposure to infectious agent, an important consideration when screening large numbers of compounds. Briefly, cell monolayers will be cultured in 96-well plates, and incubated with low-serum media containing antiviral candidates at concentrations necessary to establish dose-response curves. DENV will be added to the wells, and the plates allowed to incubate for desired time periods, after which the monolayers will be fixed with

acetone:methanol. Cell monolayer-associated viral antigens will be detected using antibodies available for arboviruses, and quantitated by standard ELISA protocols. Duplicate plates incubated with antiviral compounds alone will be used for MTT assays to simultaneously quantitate cell viability and cytotoxic concentration (CC50). EC50 and selectivity indices (CC50/EC50) will be calculated for each antiviral. Studies have shown that this ELISA assay can readily detect anti-DENV activity. Promising antivirals will be tested in several cell lines to ensure that cell-specific effects are not observed, as has been reported for ribavirin.

Cell-based West Nile and YF flavivirus ELISA Assays. Cell-based assays for replication of West Nile virus, a virus of demonstrated importance to public health in the US, and a hemorrhagic fever virus, yellow fever virus, will also be tested by the ELISA-based assays described above for DENV. For these assays a North American strain of West Nile virus will be utilized, along with the 17D vaccine strain of YFV (selected for its lab safety profile). The former virus has been utilized extensively in these assays, whereas the latter has not, but it should also function well. The implementation of these assays and data interpretation will be as for the DENV-based ELISA assays described above.

Cell-based West Nile virus replicon assay. A West Nile virus replicon based assay has been developed that is well-suited high-throughput screening of antiviral compounds. The assay requires only forty-eight hours of contact time with the test compounds, permitting the identification of lead compounds that could cause cytotoxic effects during longer-term incubations required for visual evaluation of cpe or other types of in vitro assays used for screening libraries of antiviral compounds. The assay also eliminates essentially all biosafety concerns since no infectious virus is needed. In the context of this project, this assay provides an additional test for specificity of action of the antiviral agents. Studies shown that the anti-DENV NS3 therapeutics that are targeted for development in this example have anti-replicon activity and this assay will provide useful information as new antiviral candidate compounds are synthesized.

In vitro DENV NS3 protease inhibitor assay. Recombinant DEN2V NS3 protease activity assays have been reported (see, for example, Leung et al., J. Biol. Chem. 276:45762-45771, 2001) and will serve as the basis for in vitro

NS3 protease assays. Recombinant DEN2V NS3 protease linked through a noncleavable glycine linker to a 40-residue cofactor corresponding to NS2B will be cloned, expressed in E. coli, and purified. The *ns2B/ns3* sequences will be amplified by PCR from DEN2V (strain 16681) cDNA . Cloning, expression, and purification will follow routine procedures. Protease activity will be evaluated spectrophotometrically using peptide substrates appended to p-nitroanilide chromophores; substrates will be produced by routine solid phase synthesis. Inhibition assays will be performed in 96-well plate format, and Ki values calculated using Graphpad Prism software (Hays and Watowich, J. Biol. Chem. 278:27456-27463, 2003).

Cell-based NS3 protease activity assay. Inhibition of DEN2V NS3 protease in cell culture will be monitored by examining release of secreted alkaline phosphatase protein (SEAP) from cells stably expressing the DEN2V NS2B/NS3/NS4A/SEAP polyprotein. Analogous cell-based assays have been constructed to successfully monitor HCV protease activity (Cho et al., J Virol Methods 72:109-15, 1998; Kakiuchi et al., Comb Chem High Throughput Screen. 6:155-60, 2003). The *ns2b/ns3/ns4a* sequences will be amplified by PCR from DEN2V (strain 16681) cDNA, and ligated in-frame to the 5' end of *seap* gene. The *ns2b/ns3/ns4a/seap* gene will be subcloned into the retrovirus shuttle vector pFB. Using standard laboratory protocols, the retroviral shuttle vector, and plasmids pGag-Pol and pVSV-env, will be cotransfected into 293 cells, and recombinant retrovirus recovered after approximately 48 hours. Retroviruses encoding the protease reporter gene *ns2b/ns3/ns4a/seap* will efficiently (100%) infect mammalian cells, allowing the dengue protease reporter gene to stably integrate into the cell genome and be expressed by a strong CMV promoter. Dengue NS3 protease will cleave after the NS4A cleavage site, and protease activity will be monitored by SEAP activity released into culture media. Antivirals will be incubated with cells expressing the protease reporter, and reduction in SEAP levels will indicate protease inhibition. As a positive control, cells expressing SEAP under the control of the CMV promoter will be tested to ensure that antivirals are not inhibiting SEAP transcription, translation, or activity.

Small animal toxicity, pharmacokinetics (PK) and bioavailability assays. The development of dengue antivirals will follow a tier approach with a Go/No

Go decision tree to ensure effective use of funds. The tier approach allows a project team (composed of scientists from pharmacology, toxicology, pharmacokinetics and medicinal chemistry) to critically review the data collected, and increase the chances of developing successful antivirals. This approach can avoid future development challenges as compounds are ranked in the early stages of the project based on efficacy, pharmacokinetic profile, bioavailability, toxicology and chemistry.

Tier 1 will be the small scale-up and characterization of approximately 10-20 compounds. The feasibility of the future manufacture of these compounds will be assessed by chemistry manufacturing and controls experts. Scale up will allow for PK studies to be conducted. Approximately one gram of base of each compound will be made. Evaluation of synthetic chemistry pathways indicate that it will not be problematic to produce this amount of pure (greater than 98%) compound.

Tier 2 will be the screening of approximately 10-20 antivirals that have been selected based on cell culture efficacy and toxicity models. These compounds will be evaluated in pharmacokinetic studies in the Sprague-Dawley rat to determine bioavailability and PK parameters. Compounds will be ranked based on PK parameters and bioavailability. High-pressure liquid chromatographic, mass spectrometry and other equipment will be used to confirm the identity of parent compounds and metabolites, and analyze sera for any of the lead compounds that will be examined in animals, including, for example, in the rat. Since the route of administration in humans is likely to be oral, the bioavailability of these compounds is important and the following studies, among others, may be conducted: a determination of oral PK with three doses in the rat; and a determination of intravenous IV PK with three doses in the rat. Compounds with the acceptable PKs will be considered for Tier 3.

Tier 3 will be the screening of antivirals in key toxicology studies, allowing for the ranking of the compounds so that optimal antiviral candidates can be selected. The following studies, among others may be conducted: a maximal tolerated dose (MTD) oral toxicity study in the Sprague-Dawley rat; in vitro binding/ inhibition of cytochrome P-450 protein (since many drug-drug interactions are correlated with the inhibition and/or induction of cytochrome P450 enzyme activity); in vitro antimicrobial and pharmacological screens; and

reverse bacterial mutation assay. Upon completion of the Tier 3, a Go/No Go decision will be made. Compounds with the most acceptable PK and toxicology profiles will be considered for Tier 4.

Animal challenge models. Both nonhuman primates and human-cell complemented SCID mice may be used to evaluate viremia. Industry-standard procedures for cell-based assays will be used to establish antiviral efficacy and animal models to establish compound toxicity, pharmacokinetics, and bioavailability. Small animal challenge experiments may be undertaken using either a mouse viremia or SCID mouse model. In addition, West Nile virus challenge experiments in mouse models may be undertaken to test the in vivo efficacy of broad-spectrum antivirals.

Example 2

Characterization of Lead Antiviral Compounds

As described in Example 1, computer screening of virtual chemical libraries binding to DEN2V NS3 protease identified commercially available small molecules, 1,3-Bis (4-nitrophenyl)urea and 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one 10,10-dioxide monohydrate as potential NS3 inhibitors (structures shown in Fig. 7). These compounds have confirmed antiviral activity in cell culture against DEN2V and WN replicons and molecular modeling studies indicates that these antivirals interact with independent NS3 protease sites.

These two lead antivirals reduced DEN2V replication in cell culture. Two of twenty-four tested compounds (an astonishing 8% success rate) were found to be potent, selective, and non-cytotoxic inhibitors of dengue virus replication in cell culture. These antiviral compounds were predicted to bind at the P1 and catalytic sites. The twenty-four computer-predicted antiviral compounds were purchased from major chemical suppliers and their effectiveness tested in DEN2V infected LLC-MK2 cells. The antiviral assays described in Example 1, used slot-blot methods to determine relative levels of DEN2V protein production in infected LLC-MK2 cells. These assays consistently showed EUDOC-suggested compounds ARDP0011 (1,3-Bis(4-

nitrophenyl)urea) and ARDP0012 (3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one 10,10-dioxide monohydrate) reduced DEN2V replication in cell culture. These promising antivirals were unrelated to any previously reported inhibitors of dengue and hepatitis C virus replication. Following slot-blot assays, highly reproducible, robust, and sensitive cell culture antiviral activity assays were undertaken to convincingly demonstrate the potency, cytotoxicity, and selectivity of these potential antiviral compounds. Measurements of antiviral activity in cell culture were determined using independent whole cell ELISA (enzyme-linked immunosorbent assay) and foci-forming antiviral assays. Measurements of cytotoxicity were measured using a standard MTT [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] toxicity assay (Mosmann, Journal of Immunological Methods 65, 55-63, 1983).

Reduction in DEN2V replication monitored by whole cell ELISA. To reproducibly and relatively rapidly (approximately two days/compound) evaluate the efficacy of potential antiviral compounds, a dengue antiviral whole cell ELISA was developed that could be performed in a medium-throughput 96-well format. Briefly, LLC-MK2 (rhesus monkey kidney epithelial) cells were seeded in 96 well plates and incubated with DEN2V and test compound. Dose-response curves did not change if these experiments where performed by first adding DEN2V, washing the cells one hour post-infection, and then adding tested compound. Thus, the assay protocol of simultaneously adding virus and tested compound to the cells was used to simplify the liquid handling steps in the 96-well plates. After fixed time period (typically twenty-four hours, the cells were permeablized, fixed, and blocked. The presence of DEN2V proteins was monitored using mouse anti-DEN2V primary antibody, visualized by horse radish peroxidase (HRP)-conjugated anti-mouse secondary antibody, and quantitated using standard reaction protocols on an ELISA 96-well plate reader. This assay demonstrated an excellent linear response, with an R2 correlation between absorbance and number of infected cells of approximately 0.94. Graphpad Prism was used for plotting, statistical analysis, and calculation of EC50 values.

The dose-response curve for ARDP0011 (Fig. 8) was representative of the data quality obtained for ribavirin, MPA, ARDP0011, and ARDP0012. Dose-response curves showed several notable features, among them high

reproducibility, low background noise, clear plateaus at low and high compound concentrations, and excellent goodness-of-fit statistics between the data points and fit curve. Table 2 summarizes cell culture potency (EC50), cytotoxicity (CC50), and selectivity index (SI) values of tested lead compounds and controls against dengue 2 virus challenge. Significantly, EC50 values determined using a dengue whole cell ELISA for ribavirin and mycophenoic acid controls gave results that were in excellent agreement with literature values (Koff et al., Antimicrob Agents Chemother. 24:134-6, 1983; Diamond et al., Virology 304:211-21, 2002). Differences between the values observed and literature values could be due to different virus strains and cultured cells. Moreover, EC50 and SI values calculated in the two independent virus replication assays (dengue whole cell ELISA and foci-forming infectivity assays) showed excellent agreement in the measured rank-order of compound efficacy. The foci-forming assay measured production of infectious virus, and was expected to be more difficult to decrease when significant polyprotein accumulated and thus infectious dengue virus production was the rate-limiting step. Typically, though, there was agreement between dengue protein production (whole-cell ELISA) and infectious virus production (foci-forming assay).

Table 2. Measurement of cell culture potency (EC_{50}) from two independent virus replication assays (DAWCE and foci-forming) and cytotoxicity (CC_{50}) of selected antiviral compounds in LLC-MK2 cells. EC_{50} and CC_{50} values and error estimates were calculated from dose-response curves using GraphPad Prism software. Experiments were performed using dengue 2 virus and cultured LLC-MK2 cells.

Compound	cytotoxicity		dengue antiviral whole cell ELISA (DAWCE)		foci-forming antiviral assay	
	CC_{50} (uM)	CC_{50} (uM) (interpolated)	EC_{50} (uM)	SI (interpolated)	EC_{50} (uM)	SI (interpolated)
ribavirin	>100	177 ± 17	11 ± 3	16	1400 ±	0.13
MPA	>100	239 ± 16	0.23 ± 0.01	1016	4.6 ± 1	52
ARDP0011	>100	164 ± 14	0.84 ± 0.02	194	36 ± 20	4.6
ARDP0012	>100	213 ± 18	0.020 ± 0.002	10,650	0.018 ± 0.002	11,833

Significantly, the two lead compounds showed excellent and convincing antiviral activity against DEN2V in cell culture. Both lead compounds, which have not been optimized, were at least 10-fold more effective in cell culture than ribavirin. ARDP0011 had the same order of magnitude effectiveness as mycophenoic acid. ARDP0012 had an EC50 an order of magnitude lower the

mycophenolic acid, making it the most potent small molecule antiviral for dengue virus reported. CC50 values for ARDP0011 and ARDP0012 were greater than 100 micromolar (uM) (see Table 2) for experiments carried out from one to four days. Thus, both ARDP0011 and ARDP0012 had very large selectivity indices and are excellent candidates for further optimization, pharmacokinetic studies, and animal safety studies.

Reduction of infectious DEN2V monitored by foci-forming assay. The quantity of infectious particles produced is one of the most important variables for predicting pathogenesis. Cultured LLC-MK2 cells were challenged with DEN2V and increasing log concentrations of potential antiviral compound. Cells were washed, and twenty-four hours post-infection the titer of infectious DEN2V in the cell supernatant was measured as foci-forming units (ffu) per ml of cell culture. An immunohistochemical (immuno-foci) staining method was developed to measure virus titers, since standard plaque methods are notoriously difficult, unreliable, and irreproducible for DENV due to its very low cpe in cell culture. This assay was time-consuming and labor-intensive, but provided crucial independent confirmation of the potency of the lead compounds and validation of the dengue antiviral whole-cell ELISA. Briefly, the foci-forming assay followed standard plaque methods and probing the monolayer with mouse dengue 2 virus primary antibody. Foci were visualized by staining HRP-conjugated anti-mouse secondary antibody with the VIP kit (Vector Labs). Foci were easy to observe by eye, serial dilutions gave the expected foci reductions, and replicate wells gave reproducible foci counts.

The twenty-four hour post-infection dose-response curve for ARDP0012 (Fig. 9) was representative of the data quality obtained in the antiviral foci-forming assay. This dose-response curve showed high reproducibility, low background noise, clear plateaus at low and high compound concentrations, and excellent goodness-of-fit statistics to the data points. Table 2 summarizes cell culture potency (EC50) and selectivity index (SI) values obtained for the two lead compounds and controls against DEN2V with this assay. In two independent virus replication assays (dengue whole cell ELISA and foci-forming infectivity assay), ARDP0012 had low nanomolar EC50 values and SI values greater than 104. ARDP0011, MPA, and ribavirin had higher EC50 values in the foci-forming assay relative to ELISA, indicating these antiviral

compounds were less effective at reducing virus production relative to their ability to reduce viral protein production. However, rank-orders based on EC50 and SI for these two independent virus replication assays showed excellent agreement.

Lead compounds had minimal cytotoxic effects. The cytotoxicity of EUDOC-suggested lead antiviral compounds ARDP0011 and ARDP0012 was determined by MTT cell proliferation assays (Mosmann, Journal of Immunological Methods 65:55-63, 1983). This widely used assay reproducibly and quantitatively measured cytotoxic effects in cultured cells. Briefly, 96-well plates were seeded with LLC-MK2 cells and incubated with serial dilutions of ribavirin and MPA controls, ARDP0011 and ARDP0012. After fixed time (typically 24-96 hours), cells were washed and treated with MTT, and absorbance changes read on an ELISA plate reader. Assay conditions were established to minimize systematic errors (standard deviation of approximately 7%) and optimize the linear response between cell number and assay signal (goodness-of-fit R2 approximately 0.98). CC50 values were determined from dose-response curves fit to replicate data points using GraphPad Prism. As reported in Table 2, CC50 values in LLC-MK2 cells treated for twenty-four hours with ARDP0011 and ARDP0012 were 164 and 213 uM, respectively. Similar CC50 values were obtained for human hepatoma cell line Huh-7 and for cells treated 24, 48, or 96 hours. Thus, both ARDP0011 and ARDP0012 had very low cytotoxicity in cultured cells, and are thus excellent candidates for pharmacokinetic and animal safety trails. The high CC50 and SI values of the lead computer-predicted antivirals indicate they are specific inhibitors, which do not interact strongly (if at all) with host cell proteases necessary for cell survival.

Flavivirus NS3 protease binding sites are conserved. The antivirals ARDP0011 and ARDP0012 were initially selected from an extensive computer-docking calculation that predicted these compounds would bind tightly to the DEN2V NS3 protease, with ARDP0011 and ARDP0012 binding to the P1 and catalytic sites, respectively. These predictions will be further tested, and the mechanism of antiviral action conclusively shown to be NS3 inhibition. Residues surrounding the predicted ARDP0011 and ARDP0012 binding sites are highly conserved among the major clades of the flavivirus family (Table 3),

suggesting that these antivirals (or more potent derivatives) may display antiviral activity for a broad spectrum of flaviviruses. ARDP0011 was observed to have micromolar antiviral activity when tested in West Nile virus (WNV) and hepatitis C virus (HCV) subgenomic replicon cell culture assays.

Table 3. Percentage of identical NS3 residues within 8Å of the predicted antiviral binding sites are shown for dengue virus strains, Japanese encephalitis (JEV), West Nile (WNV), yellow fever (YFV), and tick-borne encephalitis (TBEV) viruses. Similar trends were noted for residues within 4Å of the binding sites, with residue identities of 90-100% for ARDP0012. There was no significant identity (<15%) between HCV and DEN2V NS3 protein sequences, although their tertiary structures could be closely aligned. Lead antivirals ARDP0011 and ARDP0012 were predicted to bind to the NS3 P1 and catalytic sites, respectively.

Antiviral	Flavivirus							
	DEN2V	DEN4V	DEN1V	DEN3V	JEV	WNV	YFV	TBEV
ARDP0011	98%	88%	83%	81%	69%	70%	64%	52%
ARDP0012	100%	91%	89%	86%	69%	66%	74%	69%

Chemical properties of lead antivirals. Antivirals referenced by EUDOC database identifiers ARDP0011 and ARDP0012 are 1,3-Bis(4-nitrophenyl)urea and 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one 10,10-dioxide monohydrate, respectively (Figure 7). These compounds were readily obtained from Aldrich Chemical Co. Nitrophenyl urea (ARDP0011) is one of two equimolar components of nicarbazin, a starter feed supplement used for approximately fifty years to prevent intestinal coccidiosis in broiler chickens. An extensive review of existing toxicological and pharmacokinetic data on nicarbazin concluded that an Acceptable Daily Intake could be supported for nicarbazin given its long and widespread use in veterinary medicine with no evidence of toxic effects (WHO International Programme on Chemical Safety, Toxicological Evaluation of Certain Veternairy Drug Residues in Food, WHO Food Additives Series 41, 50th meeting of the Joint FAO/WHO Expert, Committee on Food Additives (JECFA), World Health Organization, Geneva, 1998). This provides encouraging data supporting the development of ARDP0011 into a therapeutic with an excellent safety profile. ARDP0011 and ARDP0012 conform to Lipinski's rules and have molecular weights of approximately 300 Daltons, which are consistent with the mean molecular weight of marketed oral drugs (Wenlock et al., J. Med. Chem. 46:1250-56, 2003), indicating that these compounds have excellent physiochemical properties for development as drugs.

Lead antivirals are not non-specific serine protease inhibitors. The low cytotoxicity of ARDP0011 and ARDP0012 strongly indicated that these compounds are not broad-spectrum inhibitors of host cell proteases required for cell survival and proliferation. However, it is possible that these compounds may have some activity against serine proteases not found in mammalian cells. Thus, a standard trypsin inhibition assay (Worthington Enzyme manual) was performed to determine if high concentrations of ARDP0011 or ARDP0012 interfered with trypsin activity (Fig. 10). Briefly, purified trypsin (Pierce) was incubated with N-benzoyl-D,L-arginine-4-nitroanilide HCL (Sigma), and progress of the reaction was followed by observing the absorbance of liberated p-nitroaniline at 405 nm (Fig. 10). Benzamidine (Sigma), used as a standard trypsin inhibitor control, clearly suppressed the rate of reaction, whereas ARDP0011 (24 uM) and ARDP0012 (670 uM) had no effect on the reaction within the error of the experiment (Fig. 10). Calculation of kinetic parameters (such as k_{cat}, K_m, and K_I; Hays and Watowich, J. Biol. Chem. 278, 27456-27463, 2003 Sheth and Watowich, Biochemistry 44(33):10984-93, 2005) were not performed since clearly the lead antivirals had no significant inhibitory activity against trypsin. A similar assay, using either chromogenic or fluorescent substrates, can be used to evaluate possible serine protease inhibitor activity of potential antivirals.

Focused synthetic combinatorial chemistry libraries. As shown in Fig. 11, the compound 1,3-Bis(4-nitrophenyl)urea (Fig. 11) will be used as one of the initial leads around which libraries will be built. The initial library will contain sets of compounds that represent different types of ureas. Symmetrical bisaromatic ureas, possessing groups with properties similar to nitro group (NO_2), will be synthesized. These ureas will have benzene rings substituted with NO_2 in the *ortho* and *meta* positions as well as sulfate (SO_3^-), acetate (OAc) and nitrile (CN) in the *ortho*, *meta*, and *para* positions. Both OAc and SO_3^- are electron-withdrawing groups like NO_2 and consequently will provided aromatic rings with electron densities similar to the lead compound. Additionally, their hydrogen bonding properties are similar to NO_2. While CN is electron withdrawing it possess very different steric and hydrogen bonding profiles. Other members of the library will contain symmetrical heteroaromatic groups such as imidazole, pyridine, thiazole and triazoles ureas. These

members will possess a shape and p-cloud similar to simpler aromatics while presenting additional hydrogen bonding options. In addition, two types of unsymmetrical ureas will be present in the initial library. These will be ureas with two different aromatic groups and ureas with one aromatic group and one aliphaticgroup. The unsymmetrical bisaromatic ureas will contain combinations of the aromatic groups discussed above. The aromatic/aliphatic ureas will use the aromatic groups with both acyclic and cyclic aliphatic groups.

All the necessary ureas will by synthesized from commercially available amines. There are more than two hundred amines available from Aldrich alone. This will be done using either phosgene or carbonyldiamidazole (Nieuwenhuijzen et al., Tetrahedron Lett. 39:7811-7813, 1998; Hutchins and Chapman, Tetrahedron Lett. 35:4055-8, 1994; Huang and Sun, Chem. Lett 11:271-273, 2001; Brase et al., J. Comb. Chem. 2:710-715, 2000). The symmetrical ureas can be obtained by the addition of two equivalents of an amine to a solution of phosgene or carbonyldiimidazole. The asymmetrical ureas are accessible by stepwise addition of one amine to carbonyl diamidazole followed by addition of the second amine. In the case of asymmetrical ureas the synthesis can be carried out in solution or on solid support. As initial efforts will be to synthesize only a few hundred-member libraries, most of the initial work will be done in solution. Several dozen members of the initial ARDP0011-based library have been synthesized and are available for testing. At least about 200 additional derivatives will be systematically synthesized and tested.

As presented in Fig.12, the first of the desired symmetrical ureas (#3, Fig. 12) have been synthesized by reaction of aniline derivates (#1, Fig. 12) with carbonyldiimidazole (#2, Fig. 12) (Zhang et al., J. Org. Chem. 62, 6420-6423, 1997). Following precipitation and filtration these molecules were judged to be at least 95% pure. This will be the first approach taken to synthesize libraries of symmetrical ureas. Unsymmetrical ureas are also desired. One approach to unsymmetrical ureas is to synthesize them on a solid support (Zheng and Combs, J. Comb. Chem. 4:38-43, 2002). Supported synthesis offers simplified purification of the product, and consequently the ability to automate processes, as well as the ability to use excess reagents to facilitate difficult reactions. As the initial step to perform the solid phase synthesis of ureas, a

support has been prepared (#5, Fig. 12) with one half of the urea attached to the polymer. This carbonyldiamidazole adduct (#5, Fig. 12) allows twenty different amines to be added to different batches of the support to provide different unsymmetrical ureas. The parent thioxanthone moiety (#10, Fig. 12) was synthesized by reaction of 3-bromothiophenol (#7, Fig. 12) with 2-fluorocyanobenzene (#8, Fig. 12) followed by treatment with sulfuric acid to hydrolyze the nitrile (#9, Fig. 12) and perform the Friedel-Crafts acylation in one step (Rewcastle et al., J. Med. Chem. 34:491-496, 1991; Kristensen et al., J. Org. Chem. 68:4091-4092, 2003). This was followed by oxidation (see #10, Fig. 12). Derivatives will be synthesized by conversion of the bromide to a variety of different groups using palladium catalyzed coupling reactions (Brase et al., Tetrahedron 59:885-939, 2003). One may be concerned about attempting to do this chemistry on a molecule containing sulfur. However, Feringa has shown that thioxanthones of this type readily undergo the palladium catalyzed Suzuki reaction (Schoevaars et al., J. Org. Chem. 62:4943-4948, 1997).

An initial combinatorial chemical library has been constructed around ARDP0012, the tetrazolthio-xantheneone lead antiviral (Figure 11). The approach to this library was to synthesize parent compounds with bromide in different positions around the aromatic ring. The necessary bromothiophenols are commercially available. Palladium catalyzed couplings provided access to a wide variety of different molecules. Through the use of known reactions, Suzuki and Sonogashira couplings (Kotha et al., Tetrahedron 58:9633-9695, 2002; Herrmann, The Suzuki Cross-Coupling Applied Homogeneous Catalysis with Organometallic Compounds (2nd Edition) 1:591-598, 2002; Herrmann et al., J. of Organometallic Chem. 687:229-248, 2003; Tykwinski, Evolution in the Palladium-Catalyzed Cross-Coupling of Sp- and Sp2-Hybridized Carbon Atoms Angew. Chemie, Int. Ed. 42:1566-1568, 2003) as well as the Stille and Heck reactions (Duncton and Pattenden, The Intramolecular Stille Reaction Journal of the Chemical Society, Perkin Transactions 1: Organic and Bio-Organic Chemistry, 1235-1246, 1999; de Meijere et al., J. of Organometallic Chem. 653:129-140, 2002; Link, The Intramolecular Heck Reaction Organic Reactions (New York) 60:157-534, 2002) and Hartwig-Buchwald chemistry (Maes et al., Journal of Heterocyclic Chemistry 39:535-543, 2002; Yong and Nolan, Chemtracts 16:205-227, 2003), alkynes, alkenes, ketones, aldehydes and esters

and amines can all be added to one simple bromide. As with the ureas, this chemistry can be carried out in solution or on solid support, with solution work the preferred route. Several dozen members of the initial ARDP0012-based library have been synthesized and are available for testing, as described above, and an additional approximately 200 derivatives will be systematically synthesized. The approaches being taken for both of antiviral lead compounds are readily amenable to scale up. Working in solution may facilitate the scale up of compounds that require further testing.

The lead antivirals will serve as templates for focused combinatorial chemical libraries. Compound activity and toxicity will be evaluated in cell culture, and drug-like properties (including, for example, toxicity, bioavailability, and pharmacokinetics) evaluated in animal models. Iterative cycles of synthesis, testing, and quantitative structure-activity relationship (QSAR) analysis will rapidly improve compound potency and drug-like properties, allowing us to select optimal antivirals for subsequent IND-enabling studies.

The lead antivirals will be used as templates for focused synthetic combinatorial chemical libraries and to aggressively evaluate compound activity and toxicity in cell culture, and drug-like properties in animal models. Since poor drug-like properties (such as, for example, bioavailability, pharmacokinetics, metabolism, and toxicity) are a primary reason drug leads fail to progress beyond preclinical trials, the Accelerated Preclinical Optimization Project outlined below emphasizes simultaneously optimizing drug properties and compound potency. These metrics will guide quantitative structure-activity relationship (QSAR) analysis to improve subsequent chemical libraries. Iterative cycles of synthesis, testing, and QSAR analysis will allow the selection of optimal antivirals for IND-enabling studies. The Preclinical Optimization Project is an integrated element of a comprehensive Product Development Plan to produce antiviral drugs to combat DENV following bioterrorist incidents or natural epidemics.

Accelerated Preclinical Optimization Project for Dengue Antivirals. This example will optimize novel dengue antivirals and generate optimized antivirals for IND-enabling studies. To reduce the chance that an antiviral compound might fail in clinical trials, this example will systematically evaluate

drug-like properties as part of the lead optimization process. In addition, compound stability, non-specific binding, and mechanism of action will be evaluated.

Decision Filters to Focus Antiviral Optimization. It is critical that rigorous criteria be used to efficiently and correctly reduce focused combinatorial chemical libraries to a small number of optimized antivirals readied for GLP studies, IND filing, and subsequent clinical trials. Several decision points are in place to separate compounds for continued study. Initial filtering will be based on increased potency and reduced toxicity relative to the parent antivirals. Efficacy against the four dengue serotypes and related Category B-C flaviviruses will be used to establish a combined ordinal ranking (for example, the Kruskal-Wallis test) of compound potency, with only compounds with acceptable selectivity indexes retained. A final filtering step, designed to select optimal compounds for expensive IND-enabling studies, will involve detailed review of broad-spectrum antiviral activity, cytochrome P450 interactions, and animal toxicology, pharmacokinetic, and bioavailability data. Approximately equal numbers of compounds derived from separate classes of antiviral leads will be retained at each filter point. This will maximize compound diversity and likely enable optimized antiviral drugs to be jointly administered to synergistically target independent protease inhibitor sites. Binding of antivirals to independent protease sites will be confirmed with in vitro competition assays. As observed for HIV, inhibiting multiple viral sites can significantly delay development of resistant strains.

Iterative cycles of phased compound evaluation and quantitative structure activity relationship (QSAR)-guided combinatorial library synthesis will be performed. The assays used will be based on recommendations set forth by the FDA in their Guidance of Industry Document for Antiviral Drug Development (May, 2005).

DENV selectivity indices (SI, defined as CC50/EC50) will be determined for each compound within the focused chemical libraries. The two lead compounds had highly encouraging SI values in cell culture against DEN2V, West Nile virus, and HCV, and are predicted to bind to distinct areas within the NS3 protease cleavage site. Several hundred derivatives of each lead compound will be synthesized and their ability to inhibit DENV replication

tested. EC50 values will be calculated for each compound. In parallel, a cell-based MTT assay to determine CC50 values for each tested compound. SI values will be calculated for each derivative and compared to the lead compound to discover more potent antivirals.

West Nile virus and Yellow fever virus selectivity indices (SI) will be determined for derivatives within each focused chemical library. Compounds from each chemical library that inhibit DEN2V replication will be subsequently tested for their ability to inhibit West Nile virus and yellow fever virus replication. Virus-specific SI values will be calculated for each derivative and compared to the lead compound to discover more potent broad-spectrum antivirals.

The drug-like properties of broad-spectrum antivirals will be determined within each focused chemical library. Poor drug-like properties (such as, for example, bioavailability, pharmacokinetics, metabolism, and toxicity) are the primary reason drug leads fail to progress beyond preclinical trials, thus this example stresses optimizing drug-like properties in addition to compound potency. Promising antivirals will be evaluated for solubility, cytochrome P450 inhibition, specificity, toxicity, pharmacokinetic properties, and bioavailability in animals. This information will guide development of improved antivirals for final IND-enabling studies.

Improved combinatorial chemical libraries will be synthesized, guided by QSAR analysis of compound potency and drug-like properties. These improved libraries will be evaluated by the assays described herein.

Several iterations of library synthesis and evaluation will rapidly yield optimized antivirals for final IND-enabling studies and Phase 1 clinical trials. These trials are expected to yield a well-tolerated broad-spectrum drug that can be orally administered on a daily basis to reduce the severity and progression of flavivirus infection, or used for post-exposure prophylaxis following a bioterrorist incident. As sub-toxic concentrations of the lead antivirals are active in cell culture against DENV, and WNV and HCV replicons, the success of this example is highly probable. Moreover, this example is expected to generate orthogonal classes of optimized antivirals, since the current lead compounds have different chemical structures and are predicted to target independent NS3 protease sites.

Example 3

Synthesis of nitrophenyl urea derivatives

Following the synthetic scheme outlined in Example 2, focused combinatorial chemistry libraries were built around lead compound 1,3-Bis(4-nitrophenyl)-urea (ARDP0011). Initial efforts have resulted in the identification of two derivatives. Derivative 1 (PLOR_0001_044_1) has an EC_{50} of approximately 0.8 uM and a SI of greater than 56. Derivative 2 (PLOR_0001_042_1) has an EC_{50} of approximately 3 uM and a SI of greater than 33. EC_{50} and SI were determined as described in Examples 1 and 2. Derivative 1 and Derivative 2 are shown in Fig. 13.

Symmetrical bisaromatic ureas, possessing groups with properties similar to nitrite group (NO_2) were synthesized. These ureas have benzene rings substituted with NO2 in the ortho and meta positions as well as sulfate (SO_3^-), acetate (OAc) and nitrile (CN) in the ortho, meta and para positions. Both OAc and SO_3^- are electron-withdrawing groups like NO_2 and consequently provide aromatic rings with electron densities similar to the lead compound. Additionally, their hydrogen bonding properties are similar to NO2. Other members of the library contain symmetrical heteroaromatic groups such as imidazole, pyridine, thiazole and triazoles ureas. These members possess a shape and Π-cloud similar to simpler aromatics while presenting additional hydrogen bonding options. All the necessary ureas were synthesized from commercially available amines. There are more than 200 amines available from Aldrich alone. This was done using either phosgene or carbonyldiamidazole (Nieuwenhuijzen et al., Tetrahedron Lett. 39:7811-7813, 1998; Hutchins and Chapman, Tetrahedron Lett. 35:4055-8, 1994; Huang and Sun, Chem. Lett 11:271-273, 2001; Brase et al., J. Comb. Chem. 2:710-715, 2000). The symmetrical ureas were obtained by the addition of two equivalents of an amine to a solution of phosgene or carbonyldiimidazole. The initial work was done in solution.

Example 4

A facile and expedient route to 3-chlorothioxanth-9-one-10,10-dioxide and analogues thereof

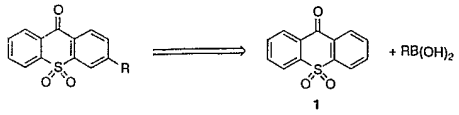
A series of novel tricyclic thioxanth-9-one-10,10-dioxide derivatives have been prepared for subsequent evaluation as anti-viral agents. A regioselective synthesis of the novel core substrate 3-chlorothioxanth-9-one-10,10-dioxide was achieved in 85% yield over three steps without the need for chromatographic purification. Subsequent microwave-assisted coupling methodology afforded the desired novel 3-substituted tricyclic compounds.

The facile assembly of therapeutically valuable molecular entities such as thioxanth-9-one-10,10-dioxides is an important medicinal chemistry goal. Thioxanthenones as well as thioxanth-9-one-10,10-dioxides have shown to possess potent anti-tumor (Azuine et al., *Pharm. Res.*, 2004, *49*, 161-169; Kostakis et al., *Med. Chem.*, 2001, 9, 2793-2802), anti-allergic (Rokach et al., US Patent 4,536,507, 1985; Rokach et al., EU Patent Appl. 0 000 978 A1, 1979; Batchelor et al., AU Patent 31113/77, 1978; Hodson et al., US Patent 4,103,015, 1978), and monoamine oxidase (Harfenist et al., *J. Med. Chem.*, 1996, 39, 1857-1863; Harfenist et al., *J. Med. Chem.*, 1994, 37, 2085-2089), (MAO) inhibitory activity. Unfortunately some of the most promising drug candidates arising from this class of compounds, such as SR23377, have show to have some adverse toxic properties in Phase I clinical trials (LoRusso et al., *Clin. Cancer Res.*, 2000, 6, 3088-3094).

The proper tuning of ADME/Tox properties of these compounds, steming from additional structure-activity relationship (SAR) data may lead to better drug candidates. Herein we report efforts to develop a versatile, expedient and efficient synthesis toward a small libray of novel thioxanth-9-one-10,10-dioxides derivatives that are now being probed for anti-viral biological activity as well as PK/PD profiling.

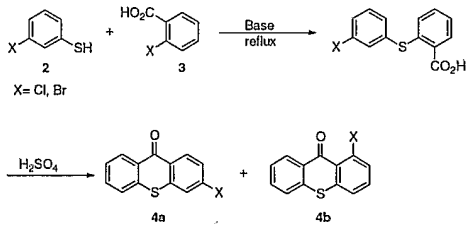
Regioselective synthesis of 3-chlorothioxanth-9-one-10,10-dioxide scaffold. The desired final 3-ary/heteroaryl thioxanth-9-one-10,10-dioxide analogues would result from a Suzuki coupling reaction between a key 3-halo-thioxanth-9-one-10,10-dioxide substrate 1 and a corresponding readily available aryl/heteroaryl boronic acid building block (Scheme 1). Consequently, the

initial goal became the development of a practical, efficient and expedient route to this core halo substrate 1.



Scheme 1

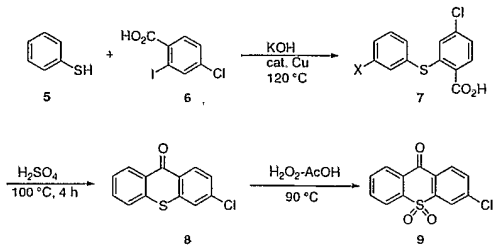
Reported syntheses for similar 3-halo-thioxanthenone ring systems utilize as first step the reaction of the corresponding *m*-halo-benzenethiol substrate 2 with 2-halo-benzoic acid 3, which, upon treatment with mineral acid, afford the desired Friedel-Crafts product 3-halo-thioxanthenone ring system 4 (Scheme 2) (Rokach et al., EU Patent Appl. 0 000 978 A1, 1979; Hodson et al., US Patent 4,103,015, 1978; Miller et al., US Patent 5,346,917, 1994; Mahishi et al., J. Karnatak Univ., 1957, 2, 50-57; Okabayashi et al., *J. Heterocyclic Chem.*, 1991, 23, 1977-1979. However, this methology invariably yields a mixture of 3-(4a) and 5-(4b) regioisomeric products that require tedious chromatographic and/or recrystallization in order to obtain either regioisomer in its pure form. Alternative protocols, although efficient, make use of both reagents and reaction conditions (*e.g.* low temperatures, pyrophoric bases such as *tert*-BuLi, longer reaction times) (Zhao et al., *Org. Lett.*, 2005, 7, 4273-4275; Kristensen et al., *J. Org. Chem.*, 2003, 68, 4091-4092; Kwon et al., *Synth. Commun.*, 2003, 33, 2437-2440), which can be uncompatible with both future scale-up and parallel synthesis.



Scheme 2

Under basic conditions the simple reaction of benzenethiol 5 with the suitably substituted 2-iodo-3-chlorobenzoic acid building block 6 in the

presence of a catalytical amount of copper (Constantino et al., *Bioorg. Med. Chem.*, 2002, 10, 3923-3931; Bonnet et al., *Bioorg. Med. Chem.*, 2000, 8, 95-103; Valenta et al., *Collect. Czech. Chem. Commun.*, 1979, 44, 2677-2688; Blanz et al., *J. Med. Chem.*, 1963, 6, 185-191) for 8h affords the desired coupled sulfide **7** in virtually quantitative yield (Scheme 3).



Scheme 3

Work-up procedure for the isolation of **7** involves simple Celite filtration of the hot reaction mixture to eliminate any adventitious traces of copper. Subsequent acidification of the filtered reaction mixture with HCl (5N) affords **7** as a white solid without the need for further purification. The presence of catalytic copper is crucial to the efficiency of this synthetic step, presumably via the in-situ formation of the corresponding cupric benzoate (Batchelor et al., AU Patent 31113/77, 1978; Hodson et al., UK Patent 1447032, 1976.

Treatment of **7** with concentrated sulfuric acid at 100°C over 4h affords the Friedel-Crafts adduct thioxanthenone 8. Upon pouring the reaction mixture onto ice, the product precipitates out as an off-white solid. No further purification is required, although copius amounts of water were vital to remove adventitious sulfuric acid.

Oxidation of 8 to the desired key sulfone substrate 9 required fine tuning of the existing oxidative protocols reported for similar deactivated aromatic ring systems (Table 4) (Balicki et al., *Prakt. Chem.*, 1999, 341, 184-18; Su, T*etrahedron Lett.*, 1994, 35, 4955-4958; Denny et al., *J. Med. Chem.*, 1991, 34, 491-496; Carpino et al., *Org. Chem.*, 1989, 54, 5887-5897; Varvoglis et al., *Chem. Res. (S)*, 1985, 186-187). The majority of the probed systems led to mixtures of sulfoxide and sulfone derivatives ranging from 45:10% to 80:5%. The effects of reaction time, temperature, variation solvent used and oxidant

stoichiometry were monitored for each oxidantion method but no significant improvement on the sulfone:sulfoxide adduct ratio was evident. Ultimately, the H_2O_2-AcOH (1:2 v/v) oxidative system (see Table 4; entry 5) by means of slow addition as well as an optimal reaction temperature of 90°C was selected to be the method of choice. Worth noting is the fact that, contrary to previously reported oxidations of similar thioxanthenone tricyclic systems (Carpino et al., *Org. Chem.*, 1989, 54, 5887-5897; Varvoglis et al., *Chem. Res. (S)*, 1985, 186-187), a 30% H_2O_2 (v/v) solution does not effect this oxidation as efficiently as the 50% H_2O_2 herein reported. In addition to being inexpensive and practical this method afforded 80% of isolated 8 and only a small amount (5%) of sulfoxide by-product when using preparative HPLC separation techniques. Moreover, sulfone 9 could be purified in a less tedious manner by recrystillization from ethyl acetate-hexanes, albeit in lower yields. This provided compound 9 in pure form as yellow needles in 70% yield.

Table 4. Selected oxidation screening conditions of 8.

entry	oxidant	Conditions	yield 8[a,b]	yield sulfoxide
1	PIFA	$CHCl_3$, RT	45%	10%
2	Oxone	DMF, 80°C	60%	10%
3	*m*-CPBA	DCM, RT	42%	15%
4	$RuCl_3$/ $NaIO_4$	CCl_4/MeCN/H_2O (1:1:2 v/v) RT	66%	5%
5	H_2O_2/ AcOH	1:2 v/v H_2O_2/AcOH, 90°C	80%	5%

[a] Reactions were carried out at 0.81 mmol of 8 and using 2.2 equiv. of oxidant as starting point whilst screening for optimal stoichiometric values. [b] Isolated yields after reverse-phase preparative HPLC.

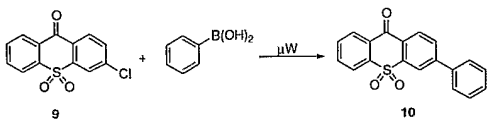
Generation of 3-substituted analogues of 9. With 9 in-hand, condictions for the microwave-assisted Suzuki coupling to give novel 3-substituted thioxanth-9-one-10,10-dioxides were screened. This effort utilized the model reaction of sulfone 9 with commercially available phenyl boronic acid (Table 5).

In-house or readily available catalytic palladium sources were used in combination different solvents, reaction temperature, base and reaction times. When using catalysts such as $Pd(dba)_3$, $Pd(OAc)_2$, $Pd(ddpf)Cl_2$, Pd/C and Fibrecat® (encapsulated palladium; John Matthey Inc.) isolated yields of coupled product were modest, ranging from 40-60%. These results were not significantly affected by variation of solvent, temperature, base used or reaction times. In all cases a substantial amount of unreacted sulfone 9 was observed and recovered.

The best results were obtained using a combination of $Pd(PPh_3)_4$as catalysts, EtOH as solvent, Cs_2CO_3 as base (1.0 M solution in H_2O) and a reaction temperature of 110°C for a period of 10 minutes (see Table 5; entry 6). The use of Cs_2CO_3 as a solution rather than a solid reagent proved to be a significant improvement in the overall methodology with the yield increasing from 65 to 87%.

The use of a polymer-supported (PS) surrogate of this catalyst system (Table 5, entry 9) resulted in an isolated yield of 78% of coupled product. In general, the use of PS-Pd catalyst systems is well known to provide cleaner reaction mixtures in coupling reactions, such as the Suzuki coupling, due to higher stability of the former to potential decomposition (Sauer et al., *Org. Lett.*, 2004, 6, 2793-2796). However, in this case simple $Pd(PPh_3)_4$ provided products of equivalent purity.

Table 5. Selected examples of protocols screened for microwave-assisted Suzuki coupling reaction.[a,b]

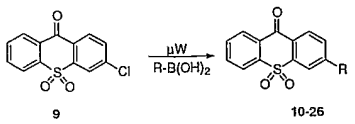


entry	Cat.	Cat. mol%	Solvent/Temp	Base	yield
1	$Pd(dba)_3$	3	DMF/155°C	K_2CO_3	40%
2	$Pd(dba)_3$	3	DMA/177°C	Cs_2CO_3	55%
3	$Pd(dba)_3$	3	EtOH/110°C	Cs_2CO_3	52%
4	$Pd(dppf)Cl_2$	10	DMF/155°C	Cs_2CO_3	56%
5	$Pd(PPh_3)_4$	3	EtOH/110°C	Cs_2CO_3	65%
6	$Pd(PPh_3)_4$	3	EtOH/110°C	1.0 M sol. Cs_2CO_3	87%
7	Pd/C	10	DMF/155°C	Cs_2CO_3	50%
8	Fibrecat®	5	DMF/155°C	Cs_2CO_3	48%
9	PS-Ph_3-Pd	10	EtOH/110°C	Cs_2CO_3	78%
10	$Pd(OAc)_2$	3	EtOH/110°C	Cs_2CO_3	60%

[a] Reactions were run using 0.089 mmol of chlorosulfone 9, 0.107 mmol of base and phenyl boronic acid and 2 mL of solvent in a CEM Discovery Reactor microwave system. A microwave irradiation power of 200 W, ramp time of 1 min with a run time of 10 mins and simultaneous cooling (PowerMax mode) was used. [b] Isolated yields are reported.

In our hands, the use of simultaneous cooling (air stream at 40 PSI) while running this microwave-assisted Suzuki coupling reaction led to a yield increase of 10 by 10% when compared to standard reaction conditions not making use of this feature (Arvela et al., *Org. Lett.*, 2005, 7, 2101-2104). Given the efficiency of the reaction it was decided that screening of additional catalytic systems known to particularly enhance coupling of less reactive chloro Suzuki substrates was not crucial and therefore necessary at this point (Chen et al., *Tetrahedron Lett.*, 2005, 46, 521; Miao et al., *J. Org. Chem.*, 2005, 70, 2332; Nájera et al., *Adv. Synth. Catal.*, 2004, 346, 1798; Navarro et al., *J. Am. Chem. Soc.*, 2003, 125, 16194-16195). For the same reason, and although it is known to often play a crucial role in the efficiency of palladium catalyzed reactions, it was deemed superfluous to proceed with the degassing of solvent used for this reaction.

Table 6. Synthesis of 17 novel 3-substituted thioxanth-9-one-10,10-dioxide analogues.



9 10-26

Product	R	Yield[a, b,c]
10		87%
11		59%
12		55%
13		30%
14		56%
15		44%
16		61%
17		80%
18		40%
19		75%
20		42%
21		43%
22		54%
23		87%
24		20%
25		53%
26		71%

[a] Reactions were run using 0.089 mmol of chlorosulfone 9, 0.089 mmol of corresponding boronic acid or boronic ester, 3 mol % $Pd(PPh_3)_4$, 0.107 mmol of Cs_2CO_3 (1.0 M solution) and 1.5 mL of EtOH. A microwave irradiation of 200 W was used he temperature being ramped from rt to 110°C in 1 min where it was then held for a total reaction time of 10 min. Simultaneous cooling was used (powermax option on CEM discover microwave unit) for all reactions. [b] Isolated yields after chromatography.[c] Unoptimized yields except for the case of 10.

The optimized conditions for the synthesis of 10 (see Table 5; entry 6) were thus utilized on the synthesis of all 17 novel analogues which were obtained in isolated yields varying from 20-87% (Table 6). We believe that both the inherent lack reactivity of 9 and the corresponding used boronic acid were the chief reason for the lower yield observed for compound 24 (Table 6). Additionally, reported low yields such as in entry 13 (Table 6) are commonly associated with electron-rich boron reagents.

In summary, a short (4 steps), efficient and practical synthesis of 17 novel thioxanth-9-one-10,10-dioxides derivatives with potential anti-viral activity has been established. Utilizing our strategy, key intermediates 8 can be easily and expediently obtained as the single regioisomer and in multi-gram quantities. Functionalization of a novel 3-chloro-thioxanth-9-one-10,10-dioxide 9 by means of microwave-assisted Suzuki coupling methodology leads to the desired adducts in yields ranging from moderate to excellent. The development of other alternative key analogues of 9 such as its 3-boronic acid congener is under way. Given the wider commercial availability of halides (versus boronic acids), the 3-boronic acid substrate will allow for the introduction of wider diverse set of functionality.

Example 5

Spectral Data for Compounds 7-26

Unless otherwise stated ^{1}H spectra were recorded on a Varian Mercury spectrometer at 300 MHz, using DMSO-d_6 or $CDCl_3$ (+ TMS) as internal reference. ^{13}C NMR spectra were recorded on the same spectrometer at 75 MHz also using DMSO-d_6 or $CDCl_3$ (+ TMS) as internal reference. LCMS analyses were conducted using a Thermo Finnigan Surveyor MSQ using (H_2O+0.05% formic acid) / (acetonitrile + 0.05% formic acid) as gradient eluants over 13 minute runs and using the following conditions: APCI dual mode; scan time=0.2 sec; cone=75 V; probe temperature=500°C; corona= 10μA with a Thermo Betabasic 8 (100 mm x 4.6 mm) column.

High-resolution mass spectrometry (ES) was performed by the Mass spectrometry & proteomics facility at the Ohio State University.

TLC analyses were performed using Merck silica gel 60 F_{254} plates purchased from EMD Chemicals Inc.

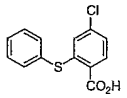
When applicable, flash chromatography was performed on a Biotage Horizon system using Biotage Si 25+M (1515-2) cartridges.

All commercially available reagents and solvents were purchased from Acros, Lancaster or Aldrich and used as received.

Microwave assisted reactions were performed on a CEM Discover™ Reactor in 10 mL reaction tubes.

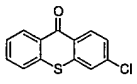
Copper powder and $Pd(PPh_3)_4$ were purchase from Aldrich.

4-chloro-2-phenylsulfanyl-benzoic acid (7)



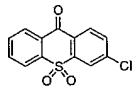
To a solution of NaOH (15.28 g; 272.0 mmol) in H_2O (180.0 mL) at RT was added 4-chloro-2-iodo benzoic acid (15.41 g; 54.50 mmol) followed by copper powder (0.346 g, 5.45 mmol) and thiophenol (5.57 mL; 54.50 mmol). The resulting reaction mixture was heated at 120 °C for 8 h and subsequently filtered through Celite while hot to remove traces of copper. The hot filtrate was cooled to RT and stirred with excess HCl (5M; 100.0 mL). The resulting white solid was filtered under reduced pressure, thoroughly washed with copious amounts of water and after transfer to a round-bottom flask where it was azeotroped with CCl_4 (100.0 mL). The title compound as a white fine powder (14.0 g, 97%). ^{1}H NMR (300 MHz, d_6-DMSO) δ 7.92 (d, 1H, J = 8.1 Hz), 7.58-7.52 (m, 5H), 7.26 (dd, 1H, J = 8.4 Hz, 8.4 Hz, 2.1 Hz), 6.52 (d, 1H, J = 2.4 Hz); ^{13}C NMR (75 MHz, d_6-DMSO) δ 167.1, 145.6, 138.0, 136.2, 133.4, 131.6, 131.0, 130.7, 126.4, 126.0, 125.2; LCMS (ESI) *m/z* (%) 265.17 (M^{-1}, 72%), 219.11 (M^{-1} - CO_2H, 100%); HRMS (ES) 286.9909 (M + Na), ($C_{13}H_9ClO_2S$ + Na requires 286.9909).

3-chloro-thioxanthen-9-one (8)



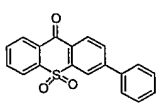
A suspension of **7** (16.18, 610.0 mmol) in conc. H_2SO_4 (160 mL) was heated to 100°C for 4h. The warm reaction mixture was poured onto ice water and subsequently stirred for 30 min. The resulting yellow precipitated was *vacuum* filtered and thoroughly washed with copious amounts of water and after transfer to a round-bottom flask where it was azeotroped with CCl_4 (3x 100 mL). This yielded the pure *title* compound (15.04, 100%) as a fine yellow powder. ^{1}H NMR (300 MHz, d_6-DMSO) δ 8.44-8.38 (m, 2H), 8.05 (d, 1H, J = 2.1 Hz), 7.84-7.74 (m, 2H), 7.58 (ddd, 2H, J = 8.4 Hz, 8.4 Hz, 2.1 Hz); ^{13}C NMR (75 MHz, d_6-DMSO) δ 178.0, 138.4, 138.1, 136.2, 133.2, 131.1, 129.0, 128.2, 127.2, 127.1, 127.0, 126.6, 125.7; LCMS (APCI) *m/z* (%) 248.85 (M^{+2}, 52), 246.85 (M, 100); HRMS (ES) 268.9809 (M + Na), ($C_{13}H_7ClOS$ + Na requires 268.9804).

3-chloro-10,10-dioxo-10*H*-10λ^6-thioxanthen-9-one (9)



To a heated solution of compound **7** (1.75 g, 7.09 mmol) in acetic acid (15 mL)
at 90°C was added drop wise H_2O_2 (30 mL; 50% wt% solution) over ½ h. The resulting reaction mixture was heated at the same temperature for 12 h. After allowing the reaction mixture to cool to RT the yellow precipitate that formed was filtered, thoroughly washed with copious amounts of H_2O (200 mL) and dried under reduced pressure. Recrystallization from EtOAc-Hexanes afforded the pure *title* compound (1.57 g; 80%) as yellow needles. ^{1}H NMR (300 MHz, d_6-DMSO) δ 8.28-8.18 (m, 4H), 8.06-7.92 (m, 3H); ^{13}C NMR (75 MHz, d_6-DMSO) δ 177.7, 142.1, 141.1, 140.5, 136.2, 134.8, 134.7, 131.8, 130.4, 129.6, 129.4, 124.0, 123.7; LCMS (APCI) *m/z* (%) 277.96 (M^{-1}, 100%); HRMS (ES) 300.9704 (M + Na), ($C_{13}H_7ClO_3S$ + Na requires 300.9702).

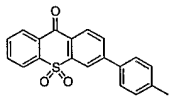
10,10-Dioxo-3-phenyl-10*H*-10λ^6-thioxanthen-9-one (10)



To a mixture of 9 (100.0 mg; 0.359 mmol), phenyl boronic acid (49.0 mg, 0.4 mmol), tetrakis (triphenylphosphine) palladium (12.4 mg; 0.01 mmol; 3.0 mol %) in ethyl alcohol (6 mL) in a 10 mL microwave tube was added Cs_2CO_3 (431.0 µl; 0.431 mmol; 1.0 M solution). The reaction mixture was then subjected to the following microwave reaction conditions: Power = 200 W; Temperature = 110°C; Time hold = 10 min.; Ramp time = 1min., Power Max on (continuous air cooling).

After allowing the reaction to cool to room temperature the contents of the microwave tube were transferred to a round-bottom flask rinsing the tube with EtOAc as needed to ensure complete residue transfer. The crude residue was then concentrated under reduced pressure, washed with brine, H_2O (3 x 5 mL) and extracted with EtOAc (3 x 10 mL). The combined organic phases were dried ($MgSO_4$), filtered through a plug of Celite and concentrated *in vacuo* to yield a beige solid. The latter was then triturated from diisopropyl ether to yield the pure *title* compound (100.0 mg; 87%) as a beige solid. ^{1}H NMR (300 MHz, d_6-DMSO) δ 8.39-8.20 (m, 5H), 8.05 (ddd, 1H, J = 7.8 Hz, 7.8 Hz, 1.2 Hz), 7.96 (ddd, 2H, J = 7.5 Hz, 7.5 Hz, 0.9 Hz), 7.90-7.86 (m, 1H), 7.60-7.50 (m, 3H); ^{13}C NMR (75 MHz, d_6-DMSO) δ 177.6, 146.6, 140.9, 140.2, 136.8, 135.5, 134.0, 131.8, 130.1, 129.9, 129.6, 129.4, 128.9, 128.7, 127.4, 123.4, 120.6; LCMS (APCI) *m/z* (%) 319.65 (M^{-1}, 100); HRMS found (ES) 343.0399 (M + Na), ($C_{19}H_{12}O_3S$ + Na requires 343.0405).

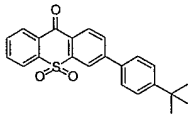
10,10-Dioxo-3-phenyl-10*H*-10λ^6-thioxanthen-9-one (11)



The title compound was synthesized employing the general reaction and work-up methodology described for the synthesis of 10 (Table 6) using compound 8 (100.0 mg; 0.359 mmol), 4-tolylboronic acid (58.4 mg; 0.43 mmol), tetrakis (triphenylphosphine) palladium (12.4 mg; 0.01 mmol; 3.0 mol %) and Cs_2CO_3 (431.0 µl; 0.431 mmol; 1.0 M solution). After the usual aqueous work-up the crude beige solid was further purified by flash chromatography (Biotage®) using EtOAc / Hexanes as eluant system (gradient of 0 to 50% over 711 mL total volume) to yield the pure *title* compound (70.0 mg; 59%) as a

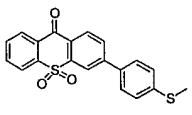
yellow solid. R_f = 0.3 (2:8 v/v EtOAc / Hexanes); ^{1}H NMR (300 MHz, d_6-DMSO) δ 8.34 (dd, 2H, J = 10.8 Hz, 1.8 Hz), 8.25 (ddd, 3H, J = 7.5 Hz, 7.5 Hz, 1.8 Hz), 8.04 (ddd, 1H, J = 7.5 Hz, 7.5 Hz, 1.2 Hz), 7.95 (ddd, 1H, J = 7.5 Hz, 7.5 Hz, 1.2 Hz), 7.79 (br d, 2H, J = 8.1 Hz), 7.35 (d, 2H, J = 8.4 Hz), 2.4 (s, 3H); ^{13}C NMR (75 MHz, d_6-DMSO) δ 178.2, 147.1, 141.6, 140.8, 140.1, 136.1, 134.6, 134.6, 132.0, 130.7, 130.7, 130.6, 129.5, 129.0, 127.8, 124.0, 120.8, 21.6; LCMS (APCI) *m/z* (%) 333.93 (M^{-1}, 100), 310 (8%); HRMS (ES) 357.0556 (M + Na), ($C_{20}H_{14}O_3S$ + Na requires 357.0561).

3-(4-*tert*-Butyl-phenyl)-10*H*-10λ^6-thioxanthen-9-one (12)



The title compound was synthesized employing the general reaction and work-up methodology described for the synthesis of 10 (Table 6) using compound 8 (100.0 mg; 0.359 mmol), 4-*tert*-butylphenylboronic acid (70.0 mg; 0.43 mmol), tetrakis (triphenylphosphine) palladium (12.4 mg; 0.01 mmol; 3.0 mol %) and Cs_2CO_3 (431.0 µl; 0.431 mmol; 1.0 M solution). After the usual aqueous work-up the crude yellow solid was further purified by flash chromatography (Biotage®) using EtOAc / Hexanes as eluant system (gradient of 0 to 50% over 711 mL total volume) to yield the pure *title* compound (75.0 mg; 55%). R_f = 0.3 (3:7 v/v EtOAc / Hexanes); ^{1}H NMR (300 MHz, d6-DMSO) δ 8.35-8.20 (m, 5H), 8.04 (ddd, 1H, J = 7.5 Hz, 7.5 Hz, 1.2 Hz), 7.95 (ddd, 1H, J = 7.5 Hz, 7.5 Hz, 1.2 Hz), 7.80 (dd, 2H, J = 8.7 Hz, 2.0 Hz), 7.55 (dd, 2H, J = 8.7 Hz, 2.0 Hz), 1.36 (s, 9H); ^{13}C NMR (75 MHz, d_6-DMSO) δ 178.2, 153.0, 147.0, 141.6, 140.8, 136.0, 134.7, 134.6, 132.1, 130.7, 130.6, 129.5, 129.1, 127.7, 126.9, 124.0, 120.9, 35.3, 31.8, 1; LCMS (APCI) m/z (%) 376.07 (M, 100) ; HRMS (ES) 399.1047 (M + Na), ($C_{19}H_{12}O_4S$ + Na requires 399.1031).

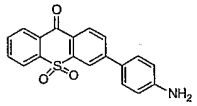
3-(4-Methoxysulfanyl-phenyl)-10*H*-10λ^6-thioxanthen-9-one (13)



The title compound was synthesized employing the general reaction and work-up methodology described for the synthesis of 10 (Table 6) using

compound 8 (100.0 mg; 0.359 mmol), 4-methylthiophenylboronic acid (72.2 mg; 0.43 mmol), tetrakis (triphenylphosphine) palladium (12.4 mg; 0.01 mmol; 3.0 mol %) and Cs_2CO_3 (431.0 µl; 0.431 mmol; 1.0 M solution). After the usual aqueous work-up the crude yellow solid was further purified by flash chromatography (Biotage®) using EtOAc / Hexanes as eluant system (gradient of 0 to 70% over 711 mL total volume) to yield the pure *title* compound (30.0 mg; 30%) as a yellow solid. R_f = 0.2 (3:7 v/v EtOAc / Hexanes);^{1}H NMR (300 MHz, $CDCl_3$) δ 8.40-8.32 (m, 2H), 8.20 (dd, 1H, J = 7.8 Hz, 0.6 Hz), 7.97-7.93 (m, 2H), 7.88 (ddd, 1H, J = 7.5 Hz, 7.5 Hz, 1.2 Hz), 7.80 (ddd, 1H, J = 7.8 Hz, 7.8 Hz, 1.2 Hz), 7.64 (dd, 2H, J = 7.8 Hz, 0.9 Hz), 7.38 (dd, 2H, J = 7.8 Hz, 0.6 Hz), 2.58 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 178.0, 147.2, 141.7, 141.4, 141.1, 134.7, 134.2, 133.4, 131.0, 130.9, 130.2, 129.3, 129.0, 127.7, 126.8, 123.7, 121.4, 15.7; LCMS (APCI) *m/z* (%) 365.97 (M^{-1}, 48), 350.92 (M^{-1} – CH_3, 100); HRMS (ES) 389.0281 (M + Na), ($C_{20}H_{14}O_3S_2$ + Na requires 389.0282).

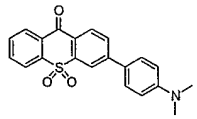
3-Amino-10*H*-10λ^6-thioxanthen-9-one (14)



The title compound was synthesized employing the general reaction and work-up methodology described for the synthesis of 10 (Table 6) using compound 8 (100.0 mg; 0.359 mmol), 4-aminophenylboronic acid (94.2 mg; 0.43 mmol), tetrakis (triphenylphosphine) palladium (12.4 mg; 0.01 mmol; 3.0 mol %) and Cs_2CO_3 (431.0 µl; 0.431 mmol; 1.0 M solution). After the usual aqueous work-up the crude red solid was further purified by flash chromatography (Biotage®) using EtOAc / Hexanes as eluant system (gradient of 0 to 70% over 711 mL total volume) to yield the pure *title* compound (67.3 mg; 56%) as a red solid. R_f = 0.2 (3:7 v/v EtOAc / Hexanes);^{1}H NMR (300 MHz, d_6-DMSO) δ 8.30-8.20 (m, 4H), 8.12 (dd, 1H, J = 8.4 Hz, 1.8 Hz), 8.02 (ddd, 1H, J = 7.8 Hz, 7.8 Hz, 1.5 Hz), 7.94 (ddd, 1H, J = 7.8 Hz, 7.8 Hz, 1.2 Hz), 7.65 (dd, 2H, J = 8.7 Hz, 2.1 Hz), 6.68 (dd, 2H, J = 8.7 Hz, 2.1 Hz), 5.73 (s, 2H); ^{13}C NMR (75 MHz, d_6-DMSO) δ 177.9, 151.5, 147.7, 141.6, 140.8, 135.9, 134.6, 130.8, 130.5, 129.9, 129.4, 128.9, 127.1, 123.9, 123.7, 118.7,

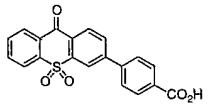
114.9; LCMS (APCI) *m/z* (%) 334.89 (M^{-1}, 100); HRMS (ES) 358.0519 (M + Na), ($C_{19}H_{13}NO_3S$ + Na requires 358.0514).

3-Dimethylamino-10*H*-10λ^6-thioxanthen-9-one (15)



The title compound was synthesized employing the general reaction and work-up methodology described for the synthesis of 10 (Table 6) using compound 8 (100.0 mg; 0.359 mmol), 4-(N,N-dimethylamino) phenylboronic acid (70.9 mg; 0.43 mmol), tetrakis (triphenylphosphine) palladium (12.4 mg; 0.01 mmol; 3.0 mol %) and Cs_2CO_3 (431.0 µl; 0.431 mmol; 1.0 M solution). After the usual aqueous work-up the crude red solid was further purified by flash chromatography (Biotage®) using EtOAc / Hexanes as eluant system (gradient of 0 to 60% over 711 mL total volume) to yield the pure *title* compound (58.0 mg; 44%) as a red solid. R_f = 0.1 (2:8 v/v EtOAc / Hexanes); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.33 (ddd, 1H, J = 7.8 Hz, 7.8 Hz, 1.5 Hz), 8.31 (dd, 1H, J = 2.4 Hz, 1.8 Hz), 8.17 (dd, 1H, J = 7.8 Hz, 1.5 Hz), 7.92 (dd, 2H, J = 7.9 Hz, 1.5 Hz), 7.85 (ddd, 1H, J = 7.5 Hz, 7.5 Hz, 1.5 Hz), 7.77 (ddd, 1H, J = 7.5 Hz, 7.5 Hz, 1.5 Hz), 7.64 (dd, 2H, J = 9.0 Hz, 2.1 Hz), 6.79 (dd, 2H, J = 9.0 Hz, 2.1 Hz), 3.05 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.5, 151.0, 147.4, 141.1, 140.8, 134.0, 132.9, 130.7, 129.7, 129.3, 128.8, 127.9, 127.1, 124.3, 123.2, 119.6, 112.2, 40.3; LCMS (APCI) *m/z* (%) 363.89 (M, 30); HRMS (ES) 364.1004 (M +H), ($C_{21}H_{17}NO_3S$ + H requires 363.1007).

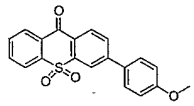
4-(9,10,10-Trioxo-9,10-dihydro-10λ^6-thioxanthen-3-yl)-benzoic acid (16)



The title compound was synthesized employing the general reaction and work-up methodology described for the synthesis of 10 (Table 6) using compound 8 (100.0 mg; 0.359 mmol), 4-carboxyphenylboronic acid (71.4 mg; 0.43 mmol), tetrakis (triphenylphosphine) palladium (12.4 mg; 0.01 mmol; 3.0 mol %) and Cs_2CO_3 (431.0 µl; 0.431 mmol; 1.0 M solution). After the usual aqueous work-up the crude yellow solid was triturated from DIPE to yield the

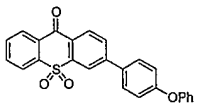
pure *title* compound (80.0 mg; 61%) as a beige solid. ^{1}H NMR (300 MHz, d_6-DMSO) δ 8.36-8.20 (m, 5H), 8.05 (ddd, 1H, J = 7.5 Hz, 7.5 Hz, 0.9 Hz), 7.98-7.92 (m, 3H), 7.80-7.74 (m, 2H); ^{13}C NMR (75 MHz, d_6-DMSO) δ 178.3,168.9, 147.4, 143.1, 141.6, 140.8, 137.0, 136.0, 134.6, 133.6, 132.2, 130.7, 130.5, 129.5, 129.1, 128.4, 126.8, 124.0, 121.0; LCMS (APCI) m/z (%) 363.96 (M^+, 20), 362.96 (M, 100), 318.94 (M-CO_2, 58); HRMS (ES) 363.0332 (M - H), ($C_{20}H_{12}O_5S$ - H requires 363.0327).

3-(4-Methoxy-phenyl)-10*H*-10λ^6-thioxanthen-9-one (17)



The title compound was synthesized employing the general reaction and work-up methodology described for the synthesis of 10 (Table 6) using compound 8 (100.0 mg; 0.359 mmol), 4-methoxy phenylboronic acid (65.3 mg; 0.43 mmol), tetrakis (triphenylphosphine) palladium (12.4 mg; 0.01 mmol; 3.0 mol %) and Cs_2CO_3 (431.0 µl; 0.431 mmol; 1.0 M solution). After the usual aqueous work-up the crude yellow solid was further purified by flash chromatography (Biotage®) using EtOAc / Hexanes as eluant system (gradient of 0 to 50% over 711 mL total volume) to yield the pure *title* compound (100.0 mg; 80%) as a yellow solid. R_f= 0.2 (2:8 v/v EtOAc / Hexanes);^{1}H NMR (300 MHz, $CDCl_3$) δ 8.37-8.30 (m, 3H), 8.18 (d, 1H, J = 7.8 Hz), 7.95-7.75 (m, 3H), 7.65 (d, 2H, J = 8.4 Hz), 7.02 (d, 2H, J = 8.4 Hz), 3.82 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.3, 156.3, 142.7, 136.9, 136.4, 129.9, 128.6, 126.2, 126.0, 125.5, 125.4, 124.5, 124.1, 123.8, 118.9, 116.4, 110.2, 51.0; LCMS (APCI) *m/z* (%) 348.97 (M^{-1}, 12), 334.87 (M - CH_3, 10); HRMS (ES) 373.0521 (M + Na), ($C_{20}H_{14}O_4S$ + Na requires 373.0511).

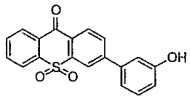
10,10-Dioxo-3-(4-phenoxy)-10*H*-10λ^6-thioxanthen-9-one (18)



The title compound was synthesized employing the general reaction and work-up methodology described for the synthesis of 10 (Table 6) using compound 8 (100.0 mg; 0.359 mmol), 4-phenoxyphenyl boronic acid (92.0 mg;

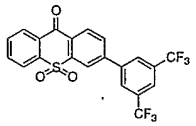
0.43 mmol), tetrakis (triphenylphosphine) palladium (12.4 mg; 0.01 mmol; 3.0 mol %) and Cs_2CO_3 (431.0 µl; 0.431 mmol; 1.0 M solution).After the usual aqueous work-up the crude yellow solid was further purified by flash chromatography (Biotage®) using EtOAc / Hexanes as eluant system (gradient of 0 to 100% over 711 mL total volume) to yield the pure *title* compound (60.0 mg; 40%) as a yellow solid. R_f = 0.2 (3:7 v/v EtOAc / Hexanes); ^{1}H NMR (300 MHz, d_6-DMSO) δ 8.34-8.19 (m, 5H), 8.04 (ddd, 1H, J = 7.2 Hz, 7.2 Hz, 1.5 Hz), 7.98-7.90 (m, 3H), 7.46-7.40 (m, 2H), 7.20 (ddd, 1H, J = 7.8 Hz, 7.8 Hz, 1.5 Hz), 7.14-7.08 (m, 4H); ^{13}C NMR (75 MHz, d_6-DMSO) δ 177.6, 158.4, 155.7, 145.9, 141.0, 140.2, 135.5, 134.0, 131.6, 131.3, 130.3, 130.1, 130.0, 129.3, 128.9, 128.4, 124.3, 123.4, 120.2, 119.5, 118.7; LCMS (APCI) m/z (%) 413.03 (M^+, 24), 412.03 (M, 100); HRMS (ES) 435.0672 (M + Na), ($C_{25}H_{16}O_4S$ + Na requires 435.0667).

3-(3-Hydroxyphenyl)-10*H*-10λ^6-thioxanthen-9-one (19)



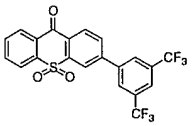
The title compound was synthesized employing the general reaction and work-up methodology described for the synthesis of 10 (Table 6) using compound 8 (100.0 mg; 0.359 mmol), 3-hydroxybenzeneboronic acid (54.4 mg; 0.43 mmol), tetrakis (triphenylphosphine) palladium (12.4 mg; 0.01 mmol; 3.0 mol %) and Cs_2CO_3 (431.0 µl; 0.431 mmol; 1.0 M solution).After the usual aqueous work-up the crude yellow solid was further purified by flash chromatography (Biotage®) using EtOAc / Hexanes as eluant system (gradient of 0 to 80% over 711 mL total volume) to yield the pure *title* compound (85.0 mg; 75%). R_f = 0.2 (1:1 v/v EtOAc / Hexanes); ^{1}H NMR (300 MHz, d6-DMSO) δ 9.78 (br. s, 1H), 8.35-8.17 (m, 5H), 8.06 (ddd, 1H, J = 7.8 Hz, 7.8 Hz, 1.2 Hz), 7.97 (ddd, 1H, J = 7.8 Hz, 7.8 Hz, 1.2 Hz), 7.39-7.27 (m, 2H), 7.20 (dd, 1H, J = 3.6 Hz, 1.9 Hz), 6.94-6.90 (m, 1H) ; ^{13}C NMR (75 MHz, d_6-DMSO) δ 177.6, 158.1, 146.6, 140.8, 140.2, 138.2, 135.4, 134.0, 131.7, 130.6, 130.1, 129.9, 128.9, 128.7, 123.4, 120.4, 118.0, 116.7, 113.9; LCMS (APCI) m/z (%) 334.91 (M^{-1}, 100) ; HRMS (ES) 359.0342 (M + Na), ($C_{19}H_{12}O_4S$ + Na requires 352.0354).

3-(3,5-Bis-trifluoromethyl-phenyl)-10*H*-10λ^6-thioxanthen-9-one (20)



The title compound was synthesized employing the general reaction and work-up methodology described for the synthesis of 10 (Table 6) using compound 8 (100.0 mg; 0.359 mmol), 3-hydroxybenzeneboronic acid (54.4 mg; 0.43 mmol), tetrakis (triphenylphosphine) palladium (12.4 mg; 0.01 mmol; 3.0 mol %) and Cs_2CO_3 (431.0 µl; 0.431 mmol; 1.0 M solution).After the usual aqueous work-up the crude yellow solid was further purified by flash chromatography (Biotage®) using EtOAc / Hexanes as eluant system (gradient of 0 to 80% over 711 mL total volume) to yield the pure *title* compound (85.0 mg; 75%). R_f = 0.2 (1:1 v/v EtOAc / Hexanes); ^{1}H NMR (300 MHz, d6-DMSO) δ 9.78 (br. s, 1H), 8.35-8.17 (m, 5H), 8.06 (ddd, 1H, J = 7.8 Hz, 7.8 Hz, 1.2 Hz), 7.97 (ddd, 1H, J = 7.8 Hz, 7.8 Hz, 1.2 Hz), 7.39-7.27 (m, 2H), 7.20 (dd, 1H, J = 3.6 Hz, 1.9 Hz), 6.94-6.90 (m, 1H) ; ^{13}C NMR (75 MHz, d_6-DMSO) δ 177.6, 158.1, 146.6, 140.8, 140.2, 138.2, 135.4, 134.0, 131.7, 130.6, 130.1, 129.9, 128.9, 128.7, 123.4, 120.4, 118.0, 116.7, 113.9; LCMS (APCI) m/z (%) 334.91 (M^{-1}, 100) ; HRMS (ES) 359.0342 (M + Na), ($C_{19}H_{12}O_4S$ + Na requires 352.0354).

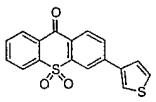
3-(3,5-Bis-trifluoromethyl-phenyl)-10*H*-10λ^6-thioxanthen-9-one (20)



The title compound was synthesized employing the general reaction and work-up methodology described for the synthesis of 10 (Table 6) using compound 8 (100.0 mg; 0.359 mmol), 3,5-bis(trifluoromethyl)phenylboronic acid (101.8 mg; 0.43 mmol), tetrakis (triphenylphosphine) palladium (12.4 mg; 0.01 mmol; 3.0 mol %) and Cs_2CO_3 (431.0 µl; 0.431 mmol; 1.0 M solution).After the usual aqueous work-up the crude beige solid was further purified by flash chromatography (Biotage®) using EtOAc / Hexanes as eluant system (gradient of 0 to 50% over 711 mL total volume) to yield the pure *title*

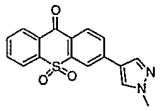
compound (68.0 mg; 42%). R_f= 0.2 (3:7 v/v EtOAc / Hexanes); ^{1}H NMR (300 MHz, d6-DMSO) δ 8.73 (d, 1H, J = 1.8 Hz), 8.58 (s, 2H), 8.43 (dd, 1H, J = 8.1 Hz, 1.8 Hz), 8.35 (d, 1H, J = 8.1 Hz), 8.30-8.20 (m, 3H), 8.05 (ddd, 1H, J = 7.5 Hz, 7.5 Hz, 1.2 Hz), 7.96 (ddd, 1H, J = 7.5 Hz, 7.5 Hz, 1.2 Hz);^{13}C NMR (75 MHz, d_6-DMSO) δ 178.3, 144.0, 141.6, 140.9, 140.2, 136.2, 134.6, 133.5, 131.9, 131.5, 130.6, 130.5, 130.4, 129.5, 129.3, 125.7, 124.0, 123.0, 122.0; LCMS (APCI) m/z (%) 455.90 (M^{-1}, 100) ; HRMS (ES) 479.0146 (M + Na), ($C_{20}H_{12}O_5S$ + Na requires 479.0153).

10,10-Dioxo-3-thiophen-3-yl-10*H*-10λ^6-thioxanthen-9-one (21)



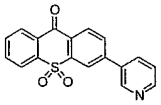
The title compound was synthesized employing the general reaction and work-up methodology described for the synthesis of 10 (Table 6) using compound 8 (100.0 mg; a.359 mmol), 2-thiopheneboronic acid (55.0 mg; 0.43 mmol), tetrakis (triphenylphosphine) palladium (12.4 mg; 0.01 mmol; 3.0 mol %) and Cs_2CO_3 (431.0 µl; 0.431 mmol; 1.0 M solution). After the usual aqueous work-up the crude dark solid was further purified by flash chromatography (Biotage®) using EtOAc / Hexanes as eluant system (gradient of 0 to 50% over 711 mL total volume) to yield the pure *title* compound (50.0 mg; 43%) as a yellow solid. R_f= 0.2 (2:8 v/v EtOAc/ Hexanes); ^{1}H NMR (300 MHz, d_6-DMSO) δ 8.35-8.17 (m, 5H), 8.04 (ddd, 1H, J = 7.8 Hz, 7.8 Hz, 1.5 Hz), 8.01 (dd, 1H, J = 3.9 Hz, 1.2 Hz), 7.96 (ddd, 1H, J = 7.8 Hz, 7.8 Hz, 1.2 Hz), 7.83 (dd, 1H, J = 5.1 Hz, 0.9 Hz), 7.26 (dd, 1H, J = 5.1 Hz, 3.9 Hz); ^{13}C NMR (75 MHz, d_6-DMSO) δ 177.8, 140.9, 140.7, 140.5, 136.0, 134.7, 130.9, 130.6, 130.5, 130.4, 130.0, 129.5, 128.8, 128.6, 124.0, 119.2; LCMS (ESI) m/z (%) 326.77 (M, 100); HRMS (ES) 348.9962 (M + Na), ($C_{17}H_{10}O_3S$ + Na requires 348.9969).

3-(1-Methyl-1*H*-pyrazol-4-yl)-10*H*-10λ^6-thioxanthen-9-one (22)



The title compound was synthesized employing the general reaction and work-up methodology described for the synthesis of 10 (Table 6) using compound 8 (100.0 mg; 0.359 mmol), 1-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-pyrazole (89.4 mg; 0.43 mmol), tetrakis (triphenylphosphine) palladium (12.4 mg; 0.01 mmol; 3.0 mol %) and Cs_2CO_3 (431.0 µl; 0.431 mmol; 1.0 M solution). After the usual aqueous work-up the crude solid was triturated from acetone to yield the *title* compound (63.0 mg; 54%) as a beige solid. ^{1}H NMR (300 MHz, d_6-DMSO) δ 8.59 (br s, 1H), 8.30-8.20 (m, 5H), 8.10 (dd, 1H, J = 8.1 Hz, 1.5 Hz), 8.02 (ddd, 1H, J = 7.5 Hz, 7.5 Hz, 1.2 Hz), 7.96 (ddd, 1H, J = 7.8 Hz, 7.8 Hz, 1.5 Hz), 3.84 (s, 3H); ^{13}C NMR (75 MHz, d_6-DMSO) δ 177.8, 141.9, 140.8, 140.5, 138.1, 135.9, 134.6, 131.0, 130.6, 130.8, 129.8, 129.4, 127.5, 124.0, 120.0, 118.9, 40.9 (hidden under DMSO signal); LCMS (APCI) *m/z* (%) 324.88 (M^+, 52); HRMS (ES) 347.0453 (M + Na), ($C_{17}H_{12}N_2O_3S$ + Na requires 347.0466).

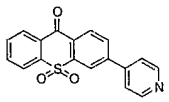
10,10-Dioxo-3-pyridin-3-yl-10*H*-10λ^6-thioxanthen-9-one (23)



The title compound was synthesized employing the general reaction and work-up methodology described for the synthesis of 10 (Table 6) using compound 8 (100.0 mg; 0.359 mmol), pyridine 3-boronic acid (52.8 mg; 0.43 mmol), tetrakis (triphenylphosphine) palladium (12.4 mg; 0.01 mmol; 3.0 mol %) and Cs_2CO_3 (431.0 µl; 0.431 mmol; 1.0 M solution). After the usual aqueous work-up the crude white solid was further purified by flash chromatography (Biotage®) using EtOAc / Hexanes as eluant system (gradient of 0 to 80% over 711 mL total volume) to yield the pure *title* compound (100.0 mg; 87%) as a white solid. R_f = 0.2 (1: 1 v/v EtOAc/ Hexanes); ^{1}H NMR (300 MHz, d_6-DMSO) δ 9.07 (d, 1H, J = 2.1 Hz), 8.68 (dd, 1H, J = 4.8 Hz, 1.2 Hz), 8.47 (d, 1H, J = 1.7 Hz), 8.36-8.22 (m, 5H), 8.05 (ddd, 1H, J = 7.8 Hz, 7.8 Hz, 0.9 Hz), 7.96 (ddd, 1H, J = 7.8 Hz, 7.8 Hz, 0.9 Hz), 7.56 (dd, 1H, J = 8.1 Hz, 4.8 Hz); ^{13}C NMR (75 MHz, d_6-DMSO) δ 177.0, 150.8, 148.7, 144.3, 141.6, 140.8, 136.1, 135.7, 134.6, 133.3, 132.7, 130.6, 130.5, 129.5, 124.7, 124.0, 121.8;

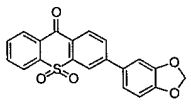
LCMS (ESI) *m/z* (%) 322.83 (M^+, 18), 321.83 (M, 100); HRMS (ES) 322.0536 (M + H), ($C_{18}H_{11}NO_3S$ + H requires 322.0538).

10,10-Dioxo-3-pyridin-4-yl-10*H*-10λ^6-thioxanthen-9-one (24)



The title compound was synthesized employing the general reaction and work-up methodology described for the synthesis of 10 (Table 6) using compound 8 (100.0 mg; 0.359 mmol), pyridine 4-boronic acid (52.8 mg; 0.43 mmol), tetrakis (triphenylphosphine) palladium (12.4 mg; 0.01 mmol; 3.0 mol %) and Cs_2CO_3 (431.0 µl; 0.431 mmol; 1.0 M solution). After the usual aqueous work-up the crude white solid was further purified by flash chromatography (Biotage®) using EtOAc / Hexanes as eluant system (gradient of 0 to 80% over 711 mL total volume) to yield the pure *title* compound (30.0 mg; 20%) as a white solid. R_f = 0.2 (1: 1 v/v EtOAc/ Hexanes); ^{1}H NMR (300 MHz, d_6-DMSO) δ 8.88-8.70 (m, 1H), 8.52 (s, 1H), 8.40-8.34 (m, 2H), 8.29 (dd, 1H, J = 7.5 Hz, 7.5 Hz, 1.2 Hz), 8.24 (dd, 1H, J = 7.5 Hz, 7.5 Hz, 1.2 Hz), 8.05 (ddd, 1H, J = 7.8 Hz, 7.8 Hz, 1.2 Hz), 7.99-7.92 (m, 3H), 7.70-7.50 (m, 1H); ^{13}C NMR (75 MHz, d_6-DMSO) δ 178.3, 151.2, 144.5, 144.3, 141.7, 140.8, 136.2, 134.7, 132.8, 130.7, 129.6, 124.1, 122.4, 121.9; LCMS (ESI) *m/z* (%) 321.56 (M, 16), 320.56 (M^{-1}, 100); HRMS (ES) 322.0540 (M + H), ($C_{18}H_{11}NO_3S$ + H requires 322.0538).

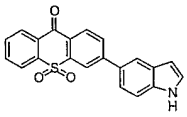
3-Benzo [1,3] dioxol-5-yl-10,10-dioxo-10*H*-10λ^6-thioxanthen-9-one (25)



The title compound was synthesized employing the general reaction and work-up methodology described for the synthesis of 10 (Table 6) using compound 8 (100.0 mg; 0.359 mmol), 3,4-(methylenedioxy)phenylboronic acid (64.7 mg; 0.43 mmol), tetrakis (triphenylphosphine) palladium (12.4 mg; 0.01 mmol; 3.0 mol %) and Cs_2CO_3 (431.0 µl; 0.431 mmol; 1.0 M solution). After the usual aqueous work-up the crude yellow solid was triturated from DIPE to yield the pure *title* compound as a yellow solid (70.0 mg; 53%); ^{1}H NMR (300

MHz, d_6-DMSO) δ 8.32-8.17 (m, 5H), 8.04 (ddd, 1H, J = 7.5 Hz, 7.5 Hz, 0.9 Hz), 7.95 (ddd, 1H, J = 7.5 Hz, 7.5 Hz, 0.9 Hz), 7.53 (d, 1H, J = 2.1 Hz), 7.42 (dd, 1H, J = 7.8 Hz, 2.1 Hz), 7.07 (d, 1H, J = 8.1 Hz), 6.10 (s, 2H); ^{13}C NMR (75 MHz, d_6-DMSO) δ 178.2, 149.4, 149.1, 146.9, 141.6, 140.9, 136.1, 134.7, 131.9, 131.6, 130.8, 130.5, 129.6, 128.9, 124.1, 122.6, 120.9, 109.8, 108.3, 102.5; LCMS (APCI) m/z (%); HRMS (ES) 387.0296 (M + Na), ($C_{20}H_{12}O_5S$ + Na requires 387.0303).

3-(1*H*-Indol-5-yl)-10,10-dioxo-10*H*-10λ^6-thioxanthen-9-one (26)



The title compound was synthesized employing the general reaction and work-up methodology described for the synthesis of 10 (Table 6) using compound 8 (100.0 mg; 0.359 mmol), 5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-indole (94.8 mg; 0.43 mmol), tetrakis (triphenylphosphine) palladium (12.4 mg; 0.01 mmol; 3.0 mol %) and Cs_2CO_3 (431.0 µl; 0.431 mmol; 1.0 M solution). After the usual aqueous work-up the crude yellow solid was further purified by flash chromatography (Biotage®) using EtOAc / Hexanes as eluant system (gradient of 0 to 100% over 711 mL total volume) to yield the pure title compound (91.7 mg; 71%). R_f = 0.2 (4:6 v/v EtOAc / Hexanes); ^{1}H NMR (300 MHz, d_6-DMSO) δ 11.31 (br. s, 1H), 8.34 (d, 1H, J = 1.8 Hz), 8.31-8.20 (m, 4H), 8.10 (br. s, 1H), 8.03 (ddd, 1H, J = 7.5 Hz, 7.5 Hz, 1.2 Hz), 7.95 (ddd, 1H, J = 7.5 Hz, 7.5 Hz, 0.9 Hz), 7.61-7.52 (m, 2H), 7.43 (dd, 1H, J = 5.1 Hz, 2.5 Hz), 6.55 (br s, 1H); ^{13}C NMR (75 MHz, d_6-DMSO) δ 178.2, 149.0, 141.5, 140.9, 137.3, 135.9, 134.6, 131.8, 130.8, 130.5, 129.4, 129.1, 128.3, 128.1, 127.6, 124.0, 121.0, 120.5, 120.2, 113.1, 102.8; LCMS (APCI) m/z (%) 358.03 (M^{-1}, 100); HRMS (ES) 358.0544 (M –H), ($C_{21}H_{13}NO_3S$ – H requires 358.0538).

The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

What is claimed is:

1. A method of treating a flavivirus infection comprising administering 1,3-Bis(4-nitrophenyl)urea, a derivative of 1,3-Bis(4-nitrophenyl)urea, 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate.

2. A method of treating a flavivirus infection comprising administering 1,3-Bis(4-nitrophenyl)urea, or a derivative thereof.

3. A method of treating a flavivirus infection comprising administering 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative thereof.

4. A method of treating a flavivirus infection in a subject, the method comprising administering 1,3-Bis(4-nitrophenyl)urea, or a derivative thereof, to a subject infected with a flavivirus in an amount effective to inhibit replication of the flavivirus.

5. A method of treating a flavivirus infection in a subject, the method comprising administering 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative thereof, to a subject infected with a flavivirus in an amount effective to inhibit replication of the flavivirus.

6. A method of preventing infection of a subject with a flavivirus, the method comprising administering 1,3-Bis(4-nitrophenyl)urea, or a derivative thereof, to the subject prior to exposure to a flavivirus.

7. A method of preventing infection of a subject with a flavivirus, the method comprising administering 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative thereof, to the subject prior to exposure to a flavivirus.

8. A method of reducing the severity of symptoms associated with a flavivirus infection, the method comprising administering 1,3-Bis(4-nitrophenyl)urea, or a derivative thereof, to the subject prior to infection with a flavivirus.

9. A method of reducing the severity of symptoms associated with a flavivirus infection, the method comprising administering 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative thereof, to the subject prior to infection with a flavivirus.

10. The method of claim 8 or 9 wherein the flavivirus infection is an infection with a dengue virus and wherein development of dengue hemorrhagic fever and/or dengue shock syndrome is prevented.

11. A method of reducing the severity of the symptoms associated with a flavivirus infection, the method comprising administering 1,3-Bis(4-nitrophenyl)urea, or a derivative thereof, to the subject after infection with a flavivirus.

12. A method of reducing the severity of the symptoms associated with a flavivirus infection, the method comprising administering 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative thereof, to the subject after infection with a flavivirus.

13. The method of claim 11 or 12 wherein the flavivirus infection is an infection with a dengue virus and wherein development of dengue hemorrhagic fever and/or dengue shock syndrome is prevented.

14. A method of inhibiting the replication of a flavivirus in a cell, the method comprising contacting cells with 1,3-Bis(4-nitrophenyl)urea, or a derivative thereof.

15. A method of inhibiting the replication of a flavivirus in a cell, the method comprising contacting cells with 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative thereof.

16. A method of identifying an agent suitable for the treatment or prevention of a flavivirus infection, the method comprising contacting cells with a flavivirus and an agent that is a derivative of 1,3-Bis(4-nitrophenyl)urea, wherein the production of a decreased flavivirus titer indicates the agent is suitable for the treatment or prevention of a flavivirus infection.

17. A method of identifying an agent suitable for the treatment or prevention of a flavivirus infection, the method comprising contacting cells with a flavivirus and an agent that is a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, wherein the production of a decreased flavivirus titer indicates the agent is suitable for the treatment or prevention of a flavivirus infection.

18. A method of identifying an agent suitable for the treatment or prevention of a flavivirus infection, the method comprising contacting a flavivirus protease with an agent that is a derivative of 1,3-Bis(4-nitrophenyl)urea, wherein an inhibition of the protease activity of the flavivirus protease indicates the agent is suitable for the treatment or prevention of a flavivirus infection.

19. A method of identifying an agent suitable for the treatment or prevention of a flavivirus infection, the method comprising contacting a flavivirus protease with an agent that is a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, wherein an inhibition of the protease activity of the flavivirus protease indicates the agent is suitable for the treatment or prevention of a flavivirus infection.

20. A method of identifying an agent suitable for the treatment or prevention of a dengue virus infection, the method comprising contacting the NS3 serine protease of a dengue virus with an agent that is a derivative of 1,3-Bis(4-nitrophenyl)urea, wherein an inhibition of the serine protease activity of the

NS3 serine protease of a dengue virus indicates the agent is suitable for the treatment or prevention of a dengue virus infection.

21. A method of identifying an agent suitable for the treatment or prevention of a dengue virus infection, the method comprising contacting the NS3 serine protease of a dengue virus with an agent that is a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, wherein an inhibition of the serine protease activity of the NS3 serine protease of a dengue virus indicates the agent is suitable for the treatment or prevention of a dengue virus infection.

22. The method of any one of claims 1-9, 11, 12, or 14-19, wherein the flavivirus is selected from the group consisting of dengue 1 virus, dengue 2 virus, dengue 3 virus, dengue 4 virus, West Nile virus, Yellow Fever virus, Japanese encephalitis virus, Murray valley fever virus, Yakose virus, Apoi virus, Rio Bravo virus, Modoc virus, Deer tick virus, Langat virus, Powassan virus, Tick-borne encephalitis virus, and Hepatitis C virus.

23. The method of any one of claims 10, 13, 20, or 21, wherein the dengue virus is selected from the group consisting of dengue 1 virus, dengue 2 virus, dengue 3 virus, and dengue 4 virus.

24. A method of treating a flavivirus infection comprising administering 1,3-Bis(4-nitrophenyl)urea, or a derivative thereof, and 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative thereof.

25. A method of preventing infection with a flavivirus, the method comprising administering 1,3-Bis(4-nitrophenyl)urea, or a derivative thereof, and 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative thereof.

26. A method of reducing the severity of the symptoms associated a flavivirus infection, the method comprising administering 1,3-Bis(4-

nitrophenyl)urea, or a derivative thereof, and 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative thereof.

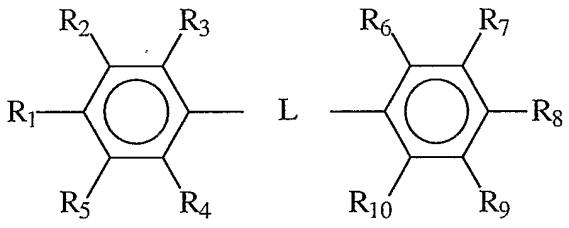
27. A method of inhibiting the replication of a flavivirus in a cell, the method comprising contacting cells with 1,3-Bis(4-nitrophenyl)urea, or a derivative thereof, and 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative thereof.

28. A derivative of 1,3-Bis(4-nitrophenyl)urea, wherein the derivative inhibits the replication of a flavivirus in cell culture.

29. The derivative of claim 28, wherein the derivative of 1,3-Bis(4-nitrophenyl)urea is an symmetrical urea derivative.

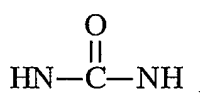
30. The derivative of claim 28, wherein the derivative of 1,3-Bis(4-nitrophenyl)urea is an asymmetrical urea derivative.

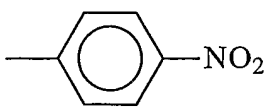
31. The derivative of claim 28, wherein the derivative of 1,3-Bis(4-nitrophenyl)urea has the structure:



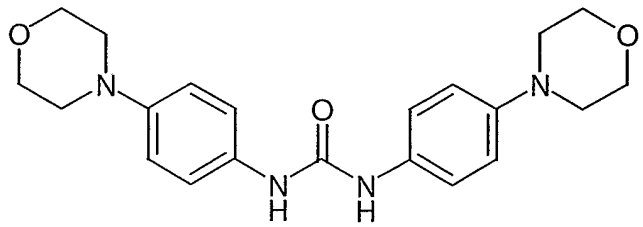
wherein L is a divalent linking group;

wherein R^1, R^2, R^3, R^4, R^5, R^6, R^7, R^8, R^9, and R^{10} are independently selected from H, F, an alkyne, an alkene, a ketone, an aldehyde, an ester or an amine; and

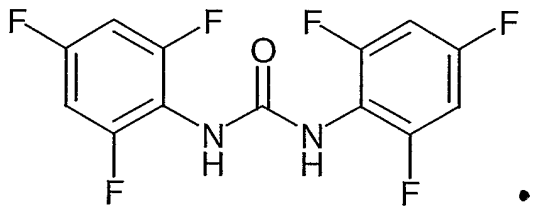
with the proviso that when L is a  moiety,

then R^1 and R^8 are not both a  moiety.

32. A compound having the formula:



33. A compound having the formula:



34. A derivative of 1,3-Bis(4-nitrophenyl)urea, wherein the derivative inhibits the replication of a dengue virus in cell culture.

35. A derivative of 1,3-Bis(4-nitrophenyl)urea, wherein the derivative inhibits the *in vivo* replication of a flavivirus.

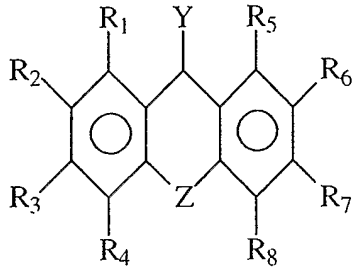
36. A derivative of 1,3-Bis(4-nitrophenyl)urea, wherein the derivative inhibits the *in vivo* replication of a dengue virus.

37. A derivative of 1,3-Bis(4-nitrophenyl)urea, wherein the derivative inhibits a flavivirus protease.

38. A derivative of 1,3-Bis(4-nitrophenyl)urea, wherein the derivative inhibits the NS3 serine protease of a dengue virus.

39. A derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, wherein the derivative inhibits the replication of a flavivirus in cell culture.

40. The derivative of claim 39 wherein the derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate has the structure:

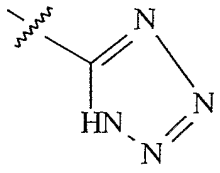


wherein Z is, SO_2, O, or a nitrogen;

wherein Y is, O, H, S, or an imine; and

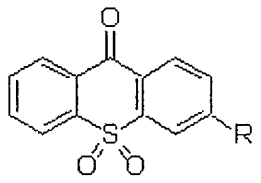
wherein each of R1 to R8 is independently selected from H, a C1-C20 organic group, an amide, or a boronic acid;

with the proviso that when Z is SO_2 and Y is O, then R7 can not be a



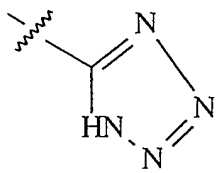
moiety.

41. The derivative of claim 39 wherein the derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate has the structure:



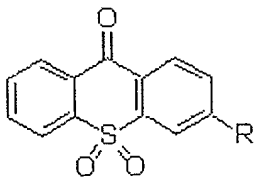
wherein R is H, a C1-C20 organic group, an amide, or a boronic acid;

with the proviso that R is not a



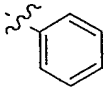
moiety.

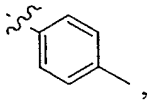
42. A compound having the structure:

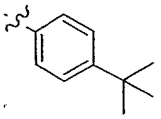


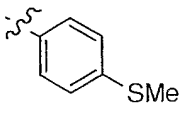
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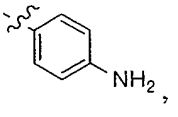
wherein R is selected from the group consisting of

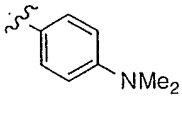
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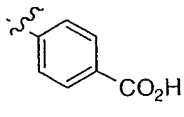
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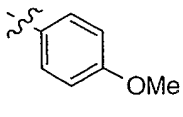
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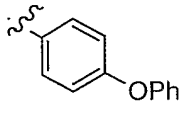
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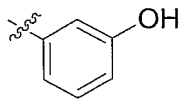
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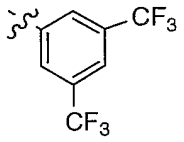
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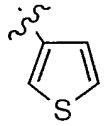
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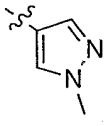
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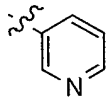
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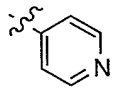
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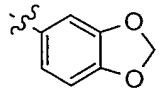
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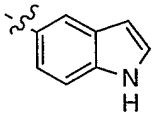
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, and



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43. A derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, wherein the derivative inhibits the replication of a dengue virus in cell culture.

44. A derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, wherein the derivative inhibits the *in vivo* replication of a flavivirus.

45. A derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, wherein the derivative inhibits the *in vivo* replication of a dengue virus.

46. A derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, wherein the derivative inhibits a flavivirus protease.

47. A derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, wherein the derivative inhibits the NS3 serine protease of a dengue virus.

48. The derivative of any one of claims 28-31, 35, 37-41, 44, or 46 wherein the flavivirus is selected from the group consisting of dengue 1 virus, dengue 2 virus, dengue 3 virus, dengue 4 virus, West Nile virus, Yellow Fever virus, Japanese encephalitis virus, Murray valley fever virus, Yakose virus, Apoi virus, Rio Bravo virus, Modoc virus, Deer tick virus, Langat virus, Powassan virus, Tick-borne encephalitis virus, and Hepatitis C virus.

49. The derivative of any one of claims 34, 36, 38, 43, 45, or 47 wherein the dengue virus is selected from the group consisting of dengue 1 virus, dengue 2 virus, dengue 3 virus, and dengue 4 virus.

50. A composition comprising a derivative of 1,3-Bis(4-nitrophenyl)urea and a pharmaceutically acceptable carrier.

51. The composition of claim 50 further comprising one or more additional antiviral agents.

52. A composition comprising of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative thereof, and a pharmaceutically acceptable carrier.

53. The composition of claim 52 further comprising one or more additional antiviral agents.

54. A composition comprising at least two agents and a pharmaceutically acceptable carrier, wherein one agent is selected from the group consisting of 1,3-Bis(4-nitrophenyl)urea, a derivative of 1,3-Bis(4-nitrophenyl)urea, 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, and a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, and wherein a second agent wherein one agent is selected from the group consisting of 1,3-Bis(4-nitrophenyl)urea, a derivative of 1,3-Bis(4-nitrophenyl)urea, 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, and a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate.

55. The composition of claim 54 further comprising one or more additional antiviral agents.

56. A bivalent agent comprising 1,3-Bis(4-nitrophenyl)urea or a derivative of 1,3-Bis(4-nitrophenyl)urea and 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate or a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate.

57. A bivalent agent comprising as one aspect 1,3-Bis(4-nitrophenyl)urea, a derivative of 1,3-Bis(4-nitrophenyl)urea, 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate or a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate and as a second aspect an additional antiviral agent.

58. A bivalent agent comprising 1,3-Bis(4-nitrophenyl)urea or a derivative of 1,3-Bis(4-nitrophenyl)urea covalently attached to 3-(1H-tetrazol-5-yl)-9H-

thio-xanthen-9-one-10,10-dioxide monohydrate or a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate.

59. A bivalent agent comprising 1,3-Bis(4-nitrophenyl)urea, a derivative of 1,3-Bis(4-nitrophenyl)urea, 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate, or a derivative of 3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one-10,10-dioxide monohydrate covalently attached to an additiaonl antiviral agent.

60. A combinatorial library comprising at least one derivative of 1,3-Bis(4-nitrophenyl)urea.

61. A combinatorial library comprising at least one derivative of 3-(1H-tetrazol-5-yl)-9H-thio xanthen-9-one-10,10-dioxide monohydrate.

62. The method of any one of claims 1-13, 22, or 23 further comprising administering one or more additional antiviral agents.

63. The methods of any one of claims 14-23 further comprising contacting the cells, flavivirus protease, or NS3 serine protease with or more additional antiviral agents.

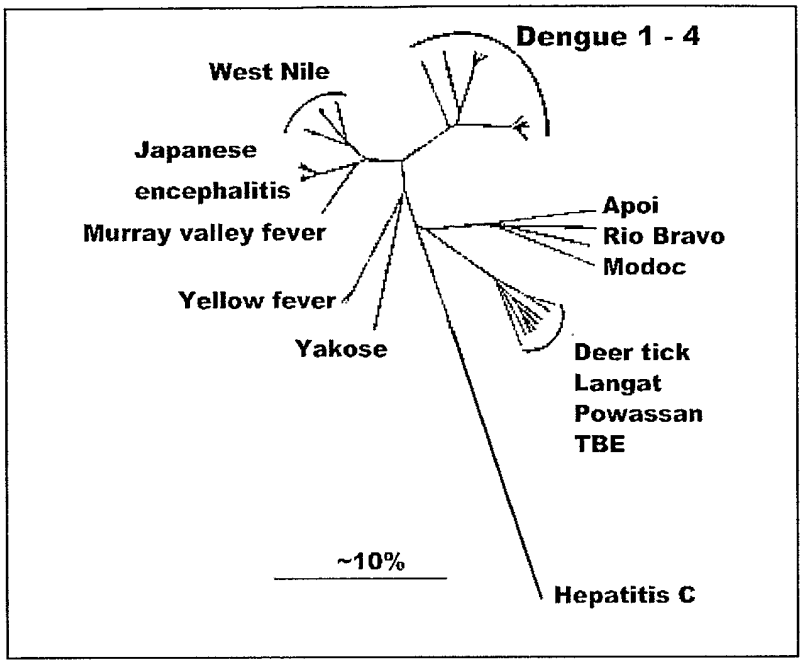

Dengue 1 - 4
West Nile
Japanese
encephalitis
Murray valley fever
Apoi
Rio Bravo
Modoc
Yellow fever
Yakose
Deer tick
Langat
Powassan
TBE
~10%
Hepatitis C

Fig. 1

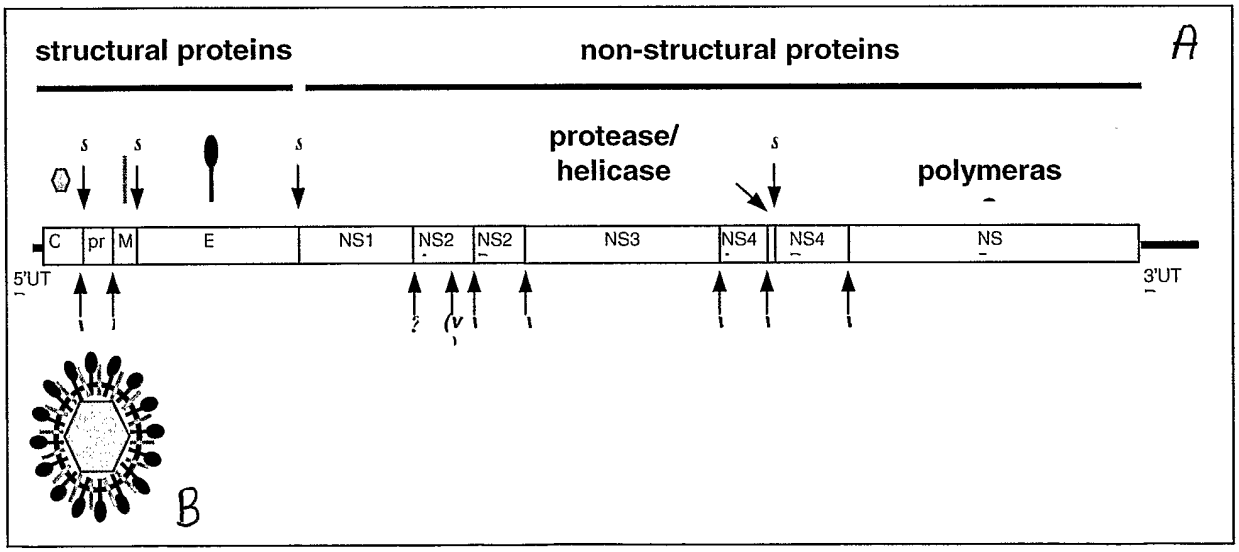

structural proteins
non-structural proteins
A
protease/
helicase
polymeras
C
pr
M
E
NS1
NS2
NS2
NS3
NS4
NS4
NS
5'UT
3'UT
B

Fig. 2

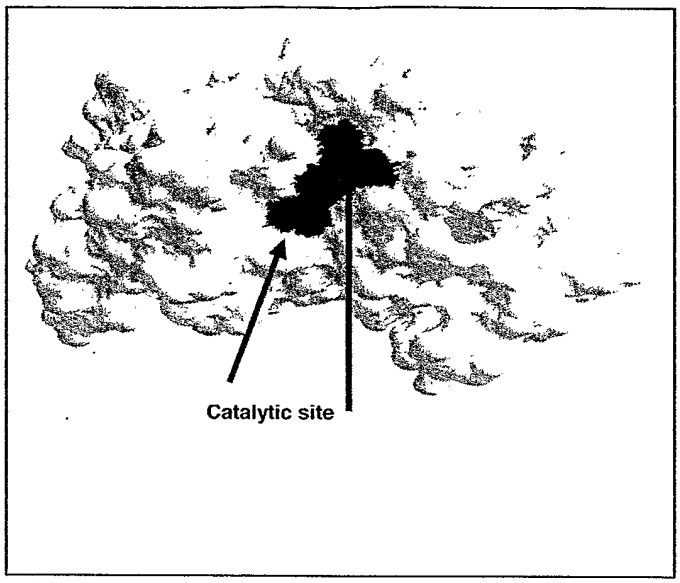

Catalytic site

Fig.3

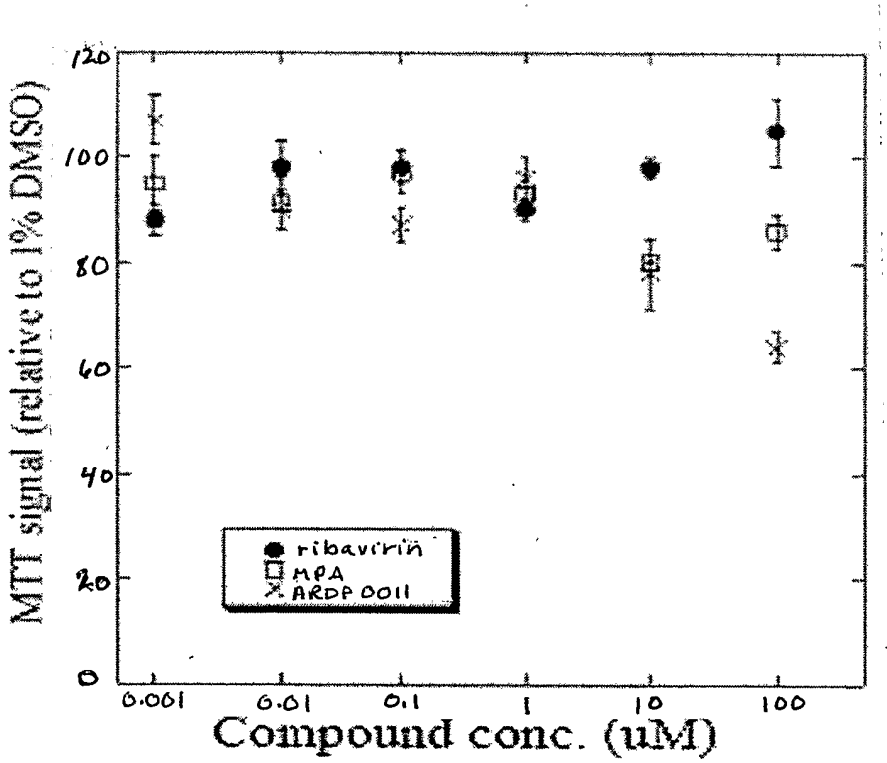

MTT signal (relative to 1% DMSO)
120
100
80
60
40
20
0
0.001
0.01
0.1
1
10
100
Compound conc. (uM)
ribavirin
MPA
ARDP0011

Fig. 4

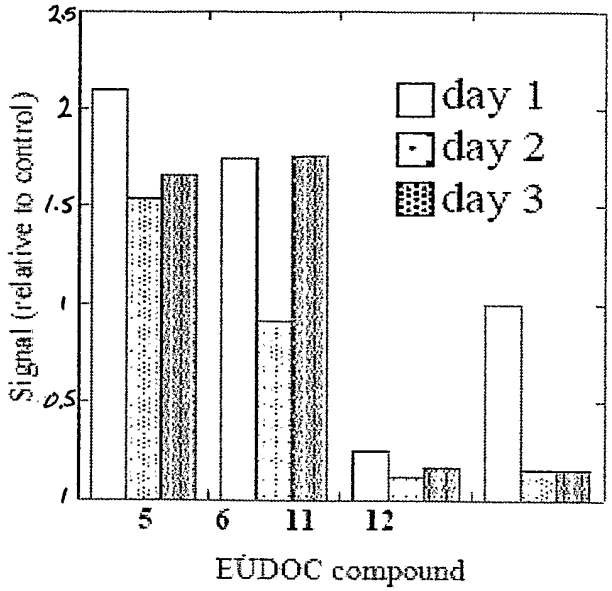

Signal (relative to control)
2.5
2
1.5
1
0.5
day 1
day 2
day 3
5
6
11
12
EUDOC compound

Fig. 5

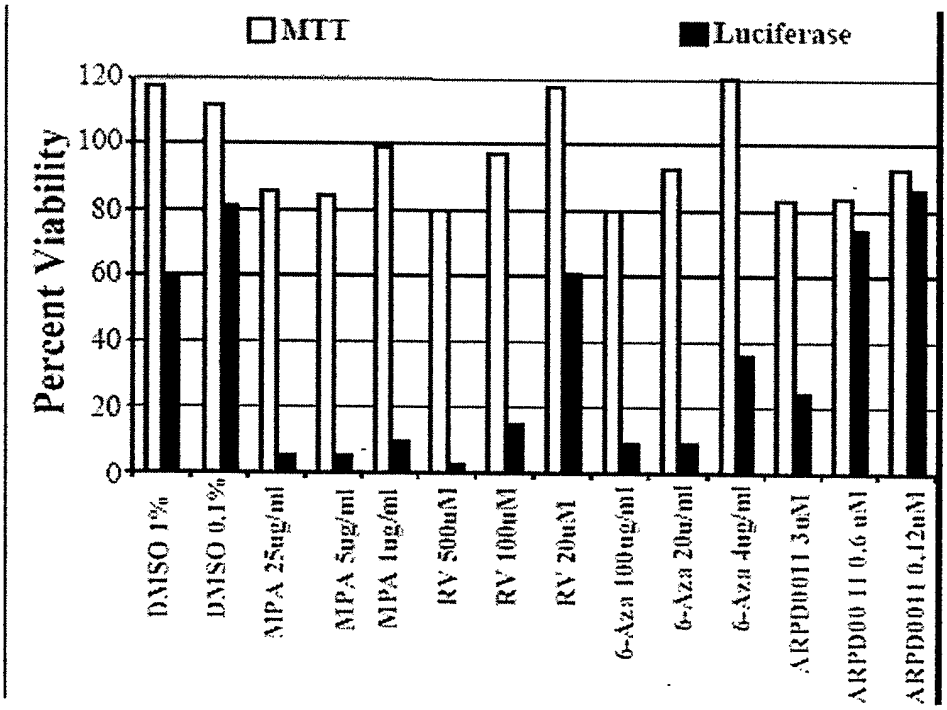

MTT
Luciferase
Percent Viability
120
100
80
60
40
20
0
DMSO 1%
DMSO 0.1%
MPA 25ug/ml
MPA 5ug/ml
MPA 1ug/ml
RV 500uM
RV 100uM
RV 20uM
6-Aza 100ug/ml
6-Aza 20u/ml
6-Aza 4ug/ml
ARPD0011 3uM
ARPD0011 0.6 uM
ARPD0011 0.12uM

Fig. 6

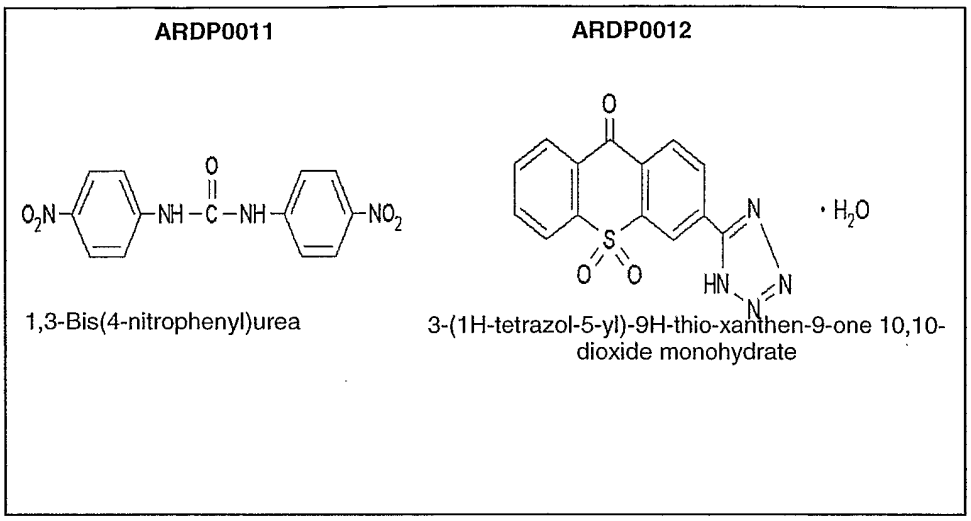

ARDP0011
ARDP0012
1,3-Bis(4-nitrophenyl)urea
3-(1H-tetrazol-5-yl)-9H-thio-xanthen-9-one 10,10-dioxide monohydrate

Fig. 7

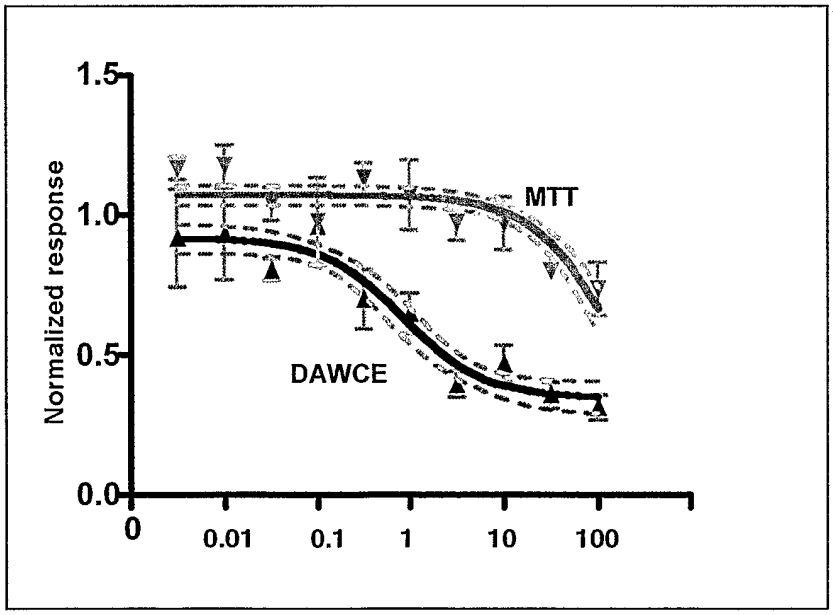

Normalized response
1.5
1.0
0.5
0.0
0
0.01
0.1
1
10
100
MTT
DAWCE

Fig. 8

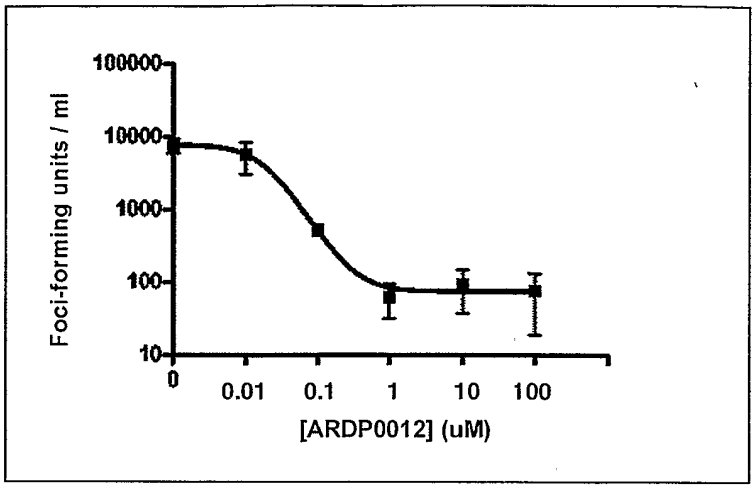

Foci-forming units / ml
100000
10000
1000
100
10
0
0.01
0.1
1
10
100
[ARDP0012] (uM)

Fig. 9

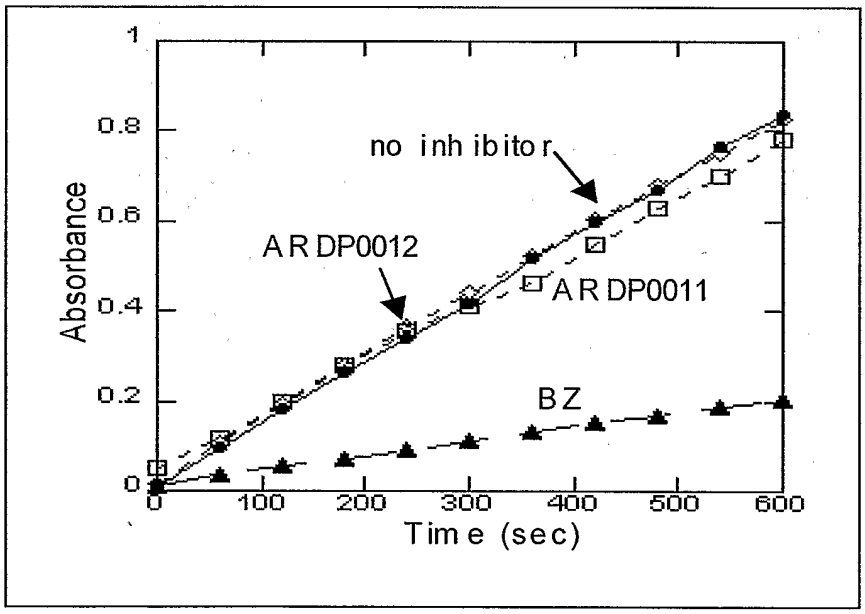

Absorbance
1
0.8
0.6
0.4
0.2
0
no inhibitor
ARDP0012
ARDP0011
BZ
0
100
200
300
400
500
600
Time (sec)

Fig. 10

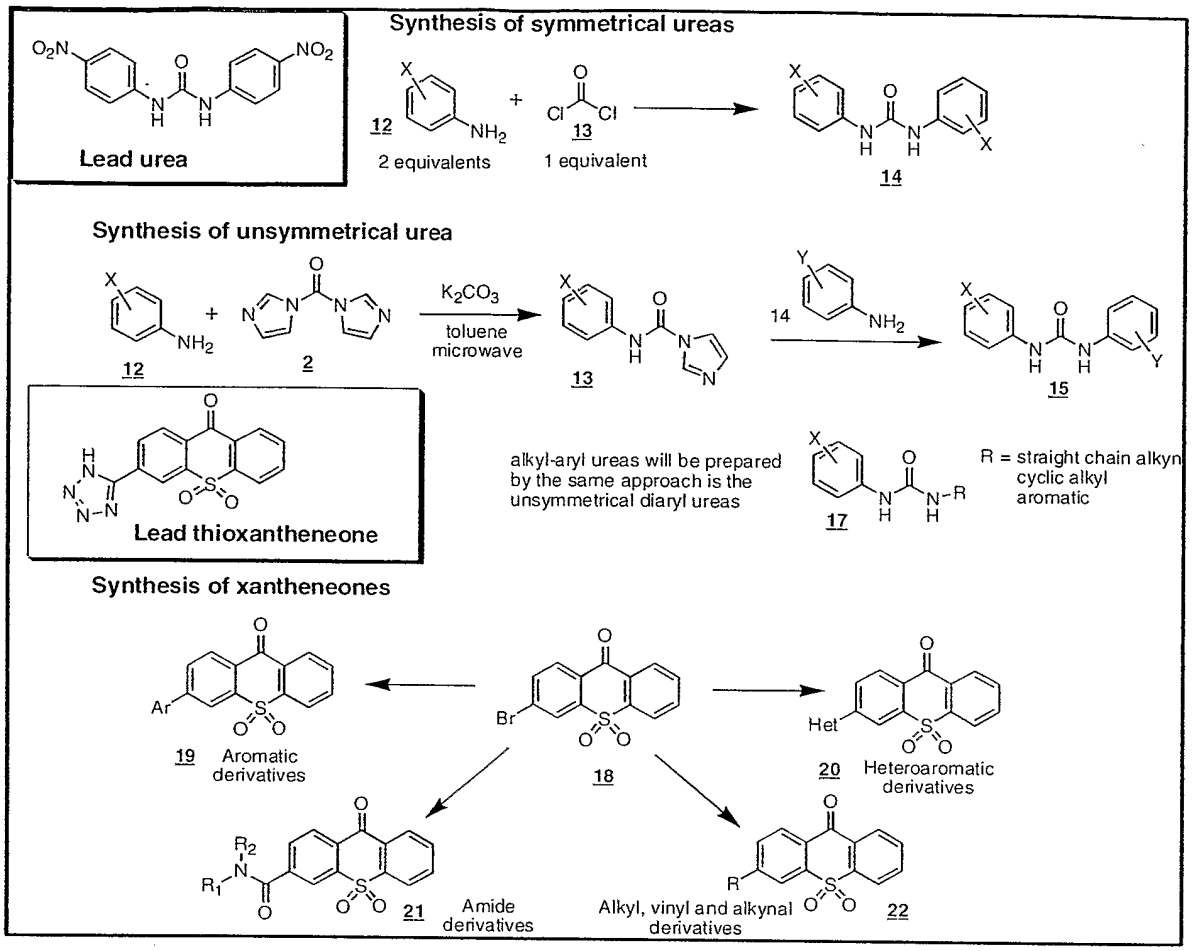

Lead urea
Synthesis of symmetrical ureas
12
2 equivalents
13
1 equivalent
14
Synthesis of unsymmetrical urea
12
2
K_2CO_3
toluene
microwave
13
14
15
Lead thioxantheneone
alkyl-aryl ureas will be prepared by the same approach is the unsymmetrical diaryl ureas
17
R = straight chain alkyn
cyclic alkyl
aromatic
Synthesis of xantheneones
19 Aromatic derivatives
18
20 Heteroaromatic derivatives
21 Amide derivatives
Alkyl, vinyl and alkynal derivatives
22

Fig. 11

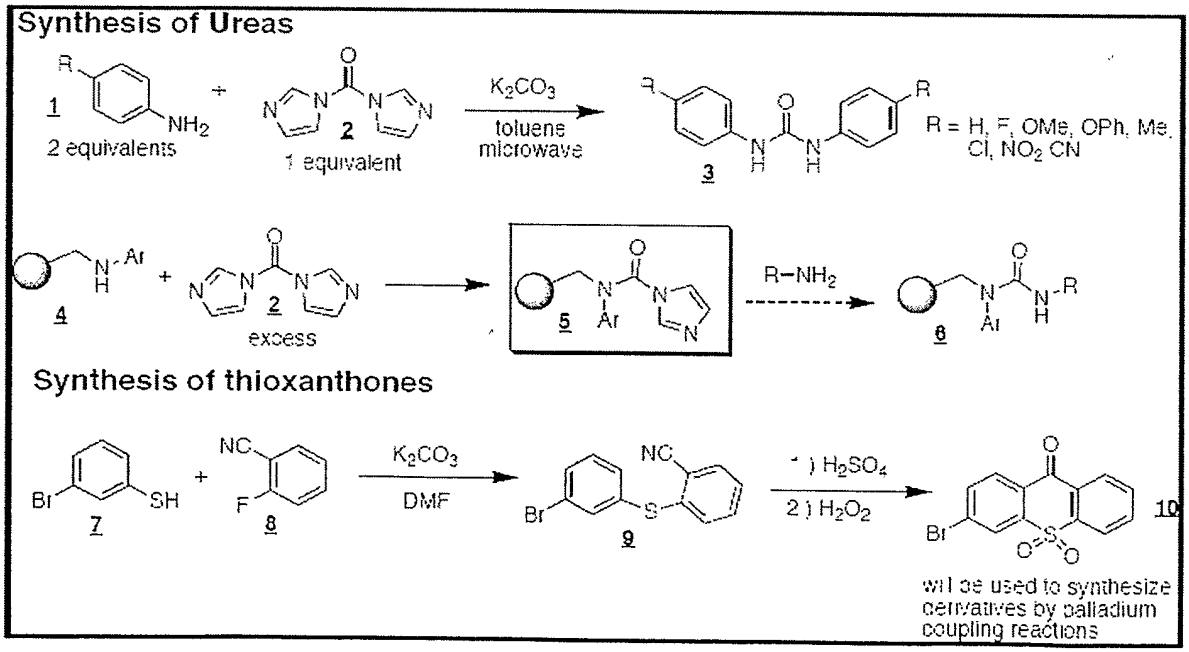
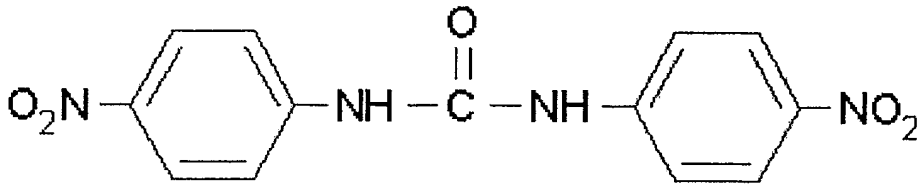

Synthesis of Ureas
1
2 equivalents
2
1 equivalent
K_2CO_3
toluene
microwave
3
R = H, F, OMe, OPh, Me, Cl, NO_2 CN
4
excess
5
$R-NH_2$
6
Synthesis of thioxanthones
7
8
K_2CO_3
DMF
9
1) H_2SO_4
2) H_2O_2
10
will be used to synthesize derivatives by palladium coupling reactions

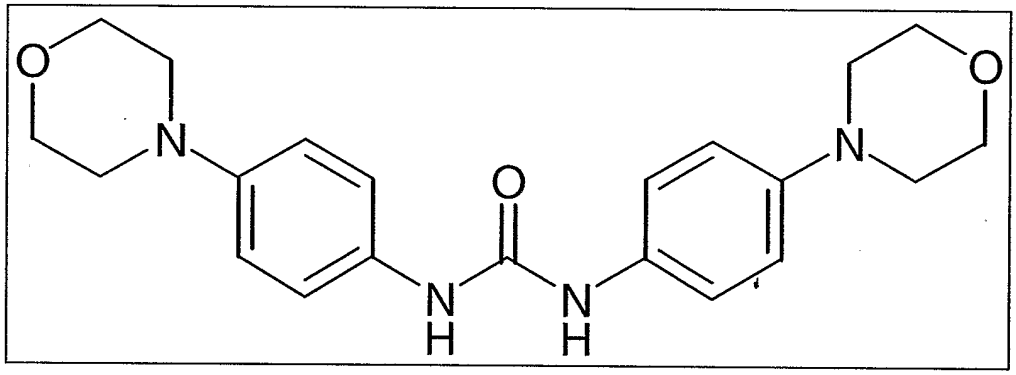
Fig. 12

Fig. 13A



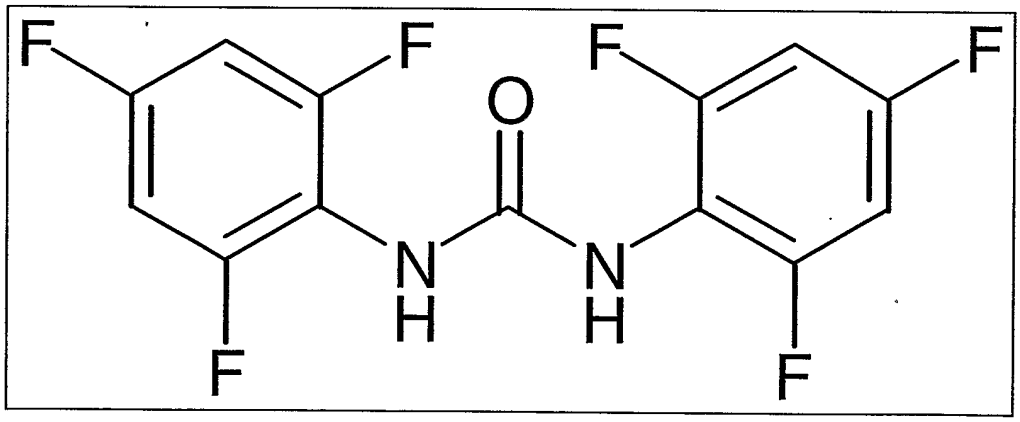
1,3-Bis(4-nitrophenyl)urea

Fig. 13B



Derivative 1

Fig. 13C



Derivative 2