

HEALTH AND MEDICINE

Discovery of direct-acting antiviral agents with a graphene-based fluorescent nanosensor

Se-Jin Park¹, Jungho Kim^{1,2}, Seounghun Kang¹, Hyung Jin Cha^{1,3}, Hojeong Shin¹, Jisang Park⁴, Yong-Suk Jang^{4,5}, Jae-Sung Woo^{3,6*}, Cheolhee Won^{2*}, Dal-Hee Min^{1,2,7*}

Direct-acting agents against viral components are considered as the most promising candidates for the successful antiviral therapeutics. To date, no direct-acting drugs exist for the treatment against dengue virus (DV) infection, which can develop into life-threatening diseases. RNA-dependent RNA polymerase (RdRp), an RNA virus-specific enzyme highly conserved among various viral families, has been known as the broad-range antiviral drug target. Here, we developed an RNA-based graphene biosensor system [RNA nano-graphene oxide system (RANGO)] to enable the fluorescence-based quantitative analysis of the RdRp enzyme activity. We used the RANGO system to a high-throughput chemical screening to identify novel direct-acting antiviral drug candidates targeting DV RdRp from the FDA-approved small-molecule library. RANGO accelerated the massive selection of drug candidates. We found that one of the selected hit compounds, montelukast, showed antiviral activity in vitro and in vivo by directly inhibiting replication of DV and thus relieved related symptoms.

INTRODUCTION

Acceleration of global outbreaks of viral diseases has become inevitable in recent years, due to rapid climate change and frequent intercontinental travel (1). Dengue virus (DV) is one of the most prevalent arthropod-borne viral pathogens in the family Flaviviridae, which also includes Zika and West Nile viruses. Currently, about 390 million people become infected by DV annually and as many as 4 billion people are at risk of potential DV infections worldwide (2). DV infection can progress into severe disease such as hemorrhagic fever and dengue shock syndrome, which can be life-threatening with rapid decline in platelet counts and severe bleeding. However, there is no specific medication for treatment of dengue infection other than pain relievers such as acetaminophen and supportive care in a hospital.

Nonselective, broad-spectrum antivirals such as interferon- γ (IFN- γ) and ribavirin are infamous for serious adverse effects including seizures and hemolytic anemia (3). Therefore, it is important to develop direct-acting antivirals to treat specific virus infections to increase therapeutic efficacy with fewer adverse effects. Strategies for development of direct-acting antiviral drugs to deal with these viruses have drawn much attention; these efforts have focused on targeting virus-originated components such as enzymes associated with the viral genomic replication (4). The main goal of this approach is to reduce the overall viral load and therefore shorten the duration of the febrile illness (5). While partial progress has been made against several flaviviruses such as hepatitis C virus (HCV) (6), there has not been successful development of direct-acting

antiviral therapeutics against DV. Although several antiviral drug candidates had entered clinical trials against DV (7, 8), these anti-DV candidates failed to show sufficient viremia reduction or NS1 (nonstructural protein 1) antigen clearance. Considering the rapid expansion of the viral outbreak, development of strategies to accelerate antiviral drug discovery is a matter of urgency.

To date, conventional in vitro assays designed for antiviral drug discovery have focused mainly on identification of target-independent antiviral activities, and restricted to cell-based assays incorporating either live virus (9–11), artificial virus-like components such as viral subgenomic replicons (12), or their mimics (13). These methods are limited to evaluation of antiviral efficacy or of partial inhibitory effects associated with the viral replication cycle, which is not well suited for high-throughput screening of direct-acting antiviral drug candidates against virus-specific biomolecular targets. In addition, these methods entail high cost, intensive labor, and complicated procedures.

Among the various RNA virus-specific enzymes, RNA-dependent RNA polymerase (RdRp) has drawn extraordinary attention as a direct-acting antiviral drug target for its critical function associated with viral genome replication and high structural conservation in numerous RNA viruses (14). However, only very limited methods exist that are compatible with high-throughput screening for detection of RdRp inhibitors as direct-acting antiviral agents. Conventional RdRp assays include gel electrophoresis or radioisotope labeling, which are not compatible with high-throughput screening due to laborious procedures and high cost.

Recent articles have also reported the development of a fluorescence-based technique that assays RdRp activity by introducing a nucleic acid–intercalating dye to distinguish double-stranded RNA (dsRNA; product of the enzyme activity) from single-stranded RNA (ssRNA; enzyme substrate). Because of its simplicity and commercial availability, there have been some efforts to perform high-throughput screening using this method (15). However, the method itself has not been inspected for its validity and suitability for the high-throughput screening. Thus, it is still uncertain whether this method guarantees the reproducibility and reliability of the screened drug candidates. Moreover, there are several concerns in using the intercalating dye, including the possibility of signal interference by the various components

¹Department of Chemistry, Seoul National University, Seoul 08826, Republic of Korea.

²Institute of Biotherapeutics Convergence Technology, Lemonex Inc., Seoul 08826, Republic of Korea. ³Center for RNA Research, Institute for Basic Science (IBS), Seoul National University, Seoul 08826, Republic of Korea. ⁴Department of Bioactive Material Sciences and Institute of Bioactive Materials, Jeonbuk National University, Jeonju 54896, Republic of Korea. ⁵Department of Molecular Biology and the Institute for Molecular Biology and Genetics, Jeonbuk National University, Jeonju 54896, Republic of Korea. ⁶Department of Life Sciences, Korea University, Seoul 02841, Republic of Korea. ⁷Department of Biological Sciences, Seoul National University, Seoul 08826, Republic of Korea.

*Corresponding author. Email: dalheemin@snu.ac.kr (D.-H.M.); cheolhee.won@lemonexbio.com (C.W.); jaesungwoo@korea.ac.kr (J.-S.W.)

other than pure nucleic acid (such as enzyme and cofactor metal ions), which may lead to false signals.

Meanwhile, graphene oxide (GO), a two-dimensional carbon-based nanomaterial with oxygen-containing functional groups, has proven its unique features suitable for sensor development. These properties include the capability of various surface functionalization chemistry, controllable selectivity toward single-stranded nucleotides, and fluorescence quenching ability (16, 17). Because of its conspicuous characteristics, GO has been used for various fluorescence-based biosensors (18–20) with high specificity. However, only very limited examples of GO-based sensors have been reported for the application to high-throughput screening for antiviral drug discovery.

Here, we developed a selective quenching fluorescent nanosensor [RNA nano-GO system (RANGO)] to enable quantitative analysis of the DV RdRp activity and its application to the high-throughput chemical screening for the rapid discovery of viral RdRp inhibitor candidates (Fig. 1). With the high specificity and robustness of GO, RANGO was experimentally validated with its reliability as a high-throughput screening platform. As a result of RANGO-based extensive screening against the 2400 Food and Drug Administration (FDA)-approved drug library, we selected an RdRp inhibitor candidate that showed the highest inhibitory property toward RNA replication. Through the series of cell- and animal-based evaluations, we propose that montelukast, primarily known as a leukotriene antagonist, could function as an antiviral drug candidate against DV by directly inhibiting viral replication.

RESULTS

Development of RANGO, an efficient DV RdRp activity analysis platform

We first developed the RdRp activity analysis technology named as RANGO based on the detection of the difference in the fluorescence signal that is proportional to the concentration of the newly synthesized RNA product mediated by active RdRp. The RANGO system consists of the recombinant DV RdRp, its target RNA template conjugated with a fluorescent dye, and nanosized GO (NGO) as a selective quencher (Fig. 1). In the RANGO system, NGO plays its role as a quencher of the fluorescence from the dye conjugated to the RNA template. The interaction between NGO and RNA is highly dependent on the structure of the substrate. In general, it is known that the interaction of NGO with the ssRNA is much stronger than that of the dsRNA (21). The strong interaction between NGO and ssRNA is based on π - π stacking interaction and hydrogen bonding, resulting in a sufficiently short distance, which induces NGO-mediated quenching of the fluorescence. When the active RdRp synthesizes a complementary RNA sequence against the template RNA, the two RNA strands form a double-stranded structure. Then, nucleobases in the dsRNA are hidden inside the helical structure, which decreases the chance of the intermolecular hydrogen bonding between NGO and RNA bases. These phenomena lead to inhibition of the fluorescence quenching that could be induced by close interaction of RNA with NGO.

We synthesized NGO by using a modified Hummers' method (19), which showed characteristic height and dimension profile corresponding to the nanosheet form of GO in atomic force microscopy analysis (fig. S1, A and B). As shown in the infrared spectra, the nanostructure consisted of the oxygen functional groups corresponding

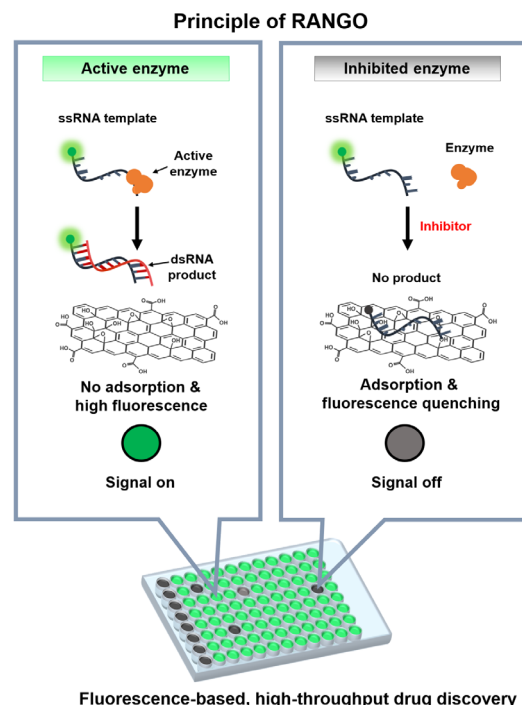


Fig. 1. Strategy for the development of the RANGO system for high-throughput screening of anti-viral drug candidates.

to each characteristic peak (fig. S1C). Together with Raman spectra analysis showing the coexistence of D- and G-band (fig. S1D), the overall analytical data indicate the successful synthesis of NGO.

For the preparation of RNA template specific to DV RdRp, overall structure, size, and the specific sequence motifs were considered. Specifically, the template was designed to include the seed sequence motif derived from 3' untranslated region (3'UTR) or 5'UTR of DV genome to guide the activation of RdRp (14). The inclusion of the secondary structure was not favorable, as the presence of the non-linear conformation in the RNA can lead to the substantial decrease of the interaction between the substrate RNA itself and NGO. These phenomena would induce the failure of the quenching of the fluorescence conjugated to the template, resulting in the false signal. In this regard, the RNA template was designed to exclude any secondary structure (fig. S2A).

Recombinant DV NS5 RdRp enzyme was obtained via the mammalian expression system. The obtained RdRp showed the expected replication activity against the designed template RNA under the appropriate buffer condition, including divalent cations (Mg^{2+} and Mn^{2+}) at specific nucleotide triphosphate (NTP) ratio of adenosine triphosphate (ATP):cytidine triphosphate (CTP):uridine triphosphate (UTP):guanosine triphosphate (GTP) = 1:5:5:5 with Hepes buffer (pH 7.5; fig. S2B). In the development of RANGO, it was critical to find the optimal range of NGO content to ensure the selective quenching property against ssRNA (representing the unreacted substrate) over dsRNA (representing the product by an enzyme). The quenching capability of NGO in various concentrations was investigated in the presence of either fluorescent label-conjugated ssRNA or the complementary dsRNA (fig. S2C). The fluorescence intensity was measured individually for each concentration of NGO. To determine the optimal NGO concentration with the highest

selective quenching capability, the difference of the fluorescence intensity (ΔFL) between ssRNA and dsRNA after addition of NGO was calculated (fig. S2D). We found that the appropriate concentration of NGO was 1.5 $\mu\text{g/ml}$ for the optimized RANGO system, which showed the highest selective quenching ability toward ssRNA over dsRNA.

To determine whether the practical operation of the RANGO system is valid, we prepared two RdRp groups, which consisted of either active RdRp or inactivated RdRp. The samples were incubated under the same condition required for the enzyme activity induction, and then the NGO was added to each sample. A simple fluorescence measurement revealed the distinct difference of the fluorescence intensity between the active RdRp group and the inactive RdRp group (Fig. 2A), indicating the reliable signal discerning capability of RANGO for the fluorescence-based RdRp activity analysis.

High-throughput screening to find inhibitors of DV RdRp by RANGO

Followed by the preliminary demonstration of the RANGO system, the platform was validated with its suitability for the high-throughput enzyme inhibitor screening assay. As the determinant standard for the reliability of assay, the Z' factor of the RANGO system was derived. The Z' factor is a generally accepted parameter used for system evaluation to determine the quality of high-throughput assay platform, of which 0.75 or higher value indicates a robust assay suitable for high-throughput screening (22). The Z' factor of the RANGO system was calculated as 0.79, suggesting that the system is reliable and suitable as a high-throughput assay (Fig. 2B). To confirm its validity for the quantitative enzyme inhibition analysis, an inhibition assay was performed with a previously known RdRp inhibitor [aurintricarboxylic acid (ATA) (23)] based on RANGO. With RANGO system, we observed the inhibitor concentration-dependent decrease of the fluorescence signal intensity, indicating that the system enabled the quantitative analysis of the model inhibitor in a dose-dependent manner (Fig. 2C).

Before the main screening, the performance of RANGO was compared with the conventional gel-based RdRp activity assay. A random chemical library consisted of 80 compounds was examined for their RdRp inhibitory effect by using either RANGO or the gel-based RNA assay. Under the experimental condition of using 96-well, black-bottom plate, a single round of RANGO system was able to analyze all the compounds in 5 to 10 min. On the contrary, a single round of the conventional gel-based RNA assay allowed up to ~20 chemicals at most and required approximately an hour (which is the summation of the time demanded for the sample transfer to gels, electrophoresis, staining, and measurement). As a result, it took roughly ~4 hours to analyze the same number of compounds with the typical gel-based assay. This comparison indicates that the utilization of RANGO system could notably reduce the time and labor required for the screening process (Fig. 2D).

With regard to the previous data, RANGO-based high-throughput RdRp inhibitor screening was performed against FDA-approved chemical library consisting of 2400 compounds (fig. S3). Throughout the first selection from the screening, we revealed the inhibitor montelukast, which showed ~90% of enzyme inhibitory efficiency at 200 μM , as the most potent RdRp inhibitor. The inhibitory property of montelukast was then quantitatively measured with various concentrations of montelukast by the RANGO system (Fig. 3A). The inhibition of RdRp activity by montelukast was revealed as

concentration dependent, showing sigmoidal curve with the calculated half-inhibitory concentration (IC_{50}) of 38.6 μM .

In vitro inhibition of DV replication by montelukast

To insist on the practical significance of RANGO to sort out the hit compound in desire, it was required to prove whether the selected RdRp inhibitor candidate also had the potential as an antiviral agent. We first designed a dose-dependent antiviral efficacy analysis based on focus-forming assay (FFA) in a model cell, VeroE6 (monkey kidney cell), infected with DV serotype 2 (Fig. 3B). Foci is a general unit for viral quantification, which is created by restricting the mobility of virus with semisolid overlay medium on cells. The focus-forming unit (FFU) was calculated individually for each group treated with the serially diluted inhibitor to derive a dose-dependent sigmoidal plot similar to that of the aforementioned enzyme inhibition assay. The half-effective concentration (EC_{50}) for the antiviral function was calculated according to the plot, with value of 5.52 μM . These data was highly correlative with the relative expression level of the viral RNA genome normalized by the endogenous control [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)], which resulted as a drug concentration-dependent decrease of the viral RNA concentration (Fig. 3C). In addition, the number of foci, which represents the severity of the viral replication and infection, significantly decreased as the concentration of the treated montelukast increases (Fig. 3D). The overall data support that montelukast not only functions as a DV RdRp inhibitor but also serves as an antiviral agent by inhibiting DV replication in the virus infection cell model.

To further validate its inhibitory activity against DV replication, we selected two human cell lines, Huh7 (24) and A549 (25), as hosts for the DV infection. Both cells were first infected with DV serotype 2 and then treated with the serially diluted montelukast. As these cells were not prone to the formation of the viral foci, the antiviral efficacy was quantified by the comparison of the viral RNA level (Fig. 3E). The treatment of the montelukast reduced the viral RNA genome level in both cell lines, dose dependently. In addition, immunostaining of the viral NS1 protein also indicated the decrease in viral replication with increasing concentration of the treated montelukast (Fig. 3F). Together, montelukast effectively reduced DV replication in two human cell lines by showing the significantly reduced level of DV RNA and viral protein NS1 upon treatment of montelukast.

In vivo antiviral effect of montelukast against DV

Encouraged by the promising data obtained in the cell-based inhibition assays, we next tested the systemic antiviral effect of montelukast in vivo. In general, $IFN-\alpha/\beta/\gamma R^{-/-}$ (AG129) mice, a transgenic mouse model highly susceptible to virus infection, were widely harnessed for infectious virus research (26, 27). Nevertheless, the sensitivity of this model varies among the different DV strains. Since the DV strain we use (KUMC-29) has not been examined for its lethality against the animal model in previous studies, it was required to investigate whether the combination of AG129 and KUMC-29 strain is appropriate for the generation of the "lethal mouse model" that we desired for in vivo efficacy study. We first examined the correlation between the quantity of the inoculated viral titer and induction of lethality. Viral infection was induced by administration of 1×10^6 , 5×10^6 , and 1×10^7 FFU of DV serotype 2 by intraperitoneal injection. According to the evaluation, the virus strain KUMC-29 showed lethality within ~10 days after administration

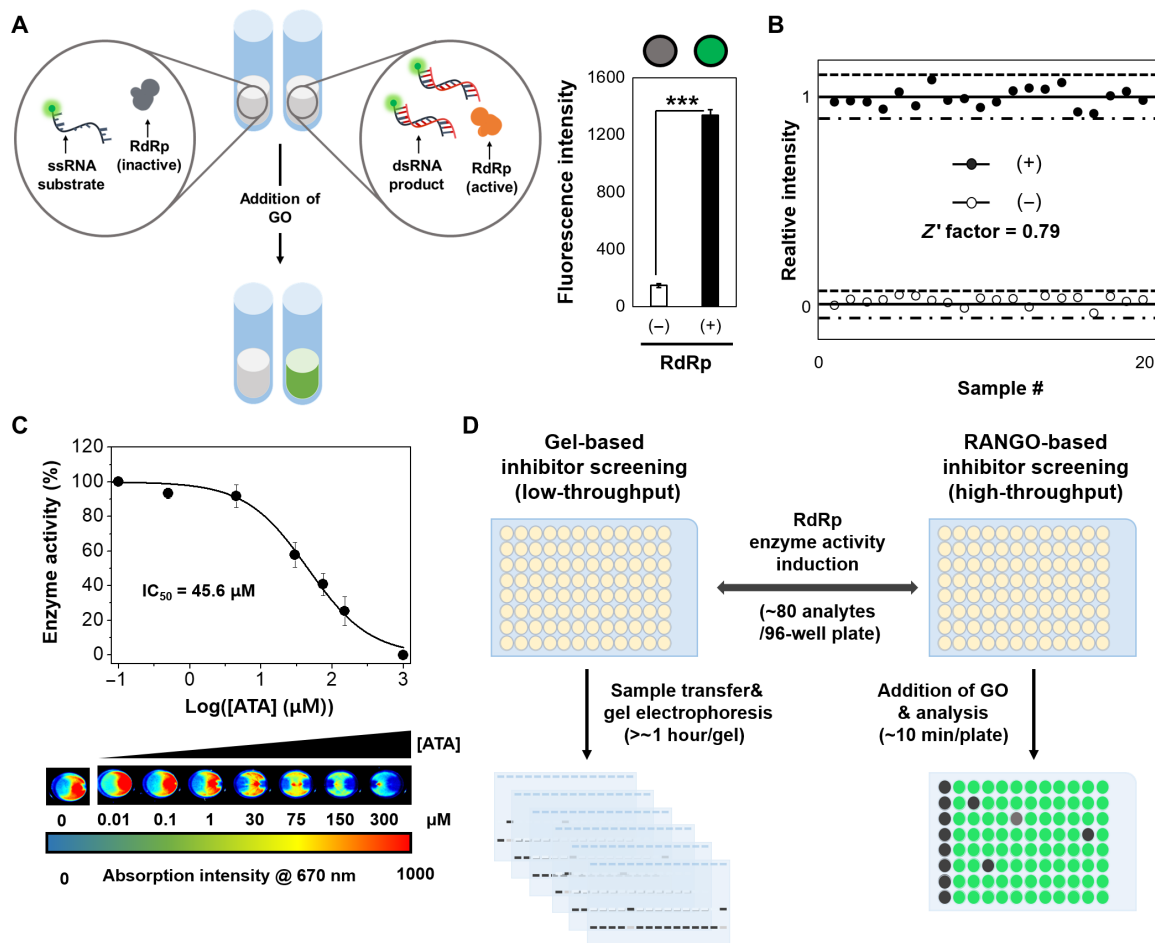


Fig. 2. DV RdRp activity assay by RANGO and its validation for the application to high-throughput RdRp inhibitor screening. (A) Schematic illustration of the RANGO-based RdRp assay platform (left) and its corresponding data depicting the difference of the fluorescence intensity between inactive RdRp and active RdRp (right). Bars indicate means $\pm$ SEM from three individual experiments for each group ($n = 3$). To determine the significance of the data, Student's t test was performed to derive P value. *** indicates a significance in the change compared to the inactive RdRp control; *** $P \leq 0.001$. (B) Significance of RANGO as a screening platform, calculated as Z' factor against the 20 individual replications ($n = 20$). (C) Fluorescence-based quantitative analysis of the representative enzyme inhibitor activity demonstrated with the RANGO system with the model inhibitor ATA in serial concentrations. Bars indicate means $\pm$ SEM from three individual groups for each concentration ($n = 3$). (D) Schematic comparison of the performance as an inhibitor drug screening platform between the conventional gel-based RNA assay and RANGO-based RdRp assay.

at a dose of $\sim 1 \times 10^7$ FFU (fig. S4). Therefore, we successfully generated the lethal DV infection mouse model by using KUMC-29 DV strain using AG129 mice.

We next tested the antiviral drug efficacy of montelukast in the DV mouse model we generated as above. Montelukast was administered by intraperitoneal injection to the lethal AG129 mouse model generated by injection of DV, in a daily dose of 10 mg/kg per day for 10 experimental days. The positive control group was daily administered with an equal volume of sterilized phosphate-buffered saline (PBS) by intraperitoneal injection. We then investigated whether the injected montelukast could alleviate the symptoms of viral infection associated with lethality. As one of the well-known symptoms, severe weight loss was observed in the lethal mouse model infected with DV before death (28). Compared to the infected control group, administration of montelukast induced the consistent maintenance of mice body weight (Fig. 4A). Next, the montelukast treatment-dependent survival probability was calculated according to the Kaplan-Meier estimate model (Fig. 4B and fig. S5). As shown in the graph, the injection of montelukast to the DV-infected mice signifi-

cantly decreased lethality and improved survival rate ($P = 0.023$). This result was highly correlative to the decreasing tendency in viremia (virus titer in plasma), indicating the inhibition of the viral replication after the systemic administration of montelukast (Fig. 4C).

When the viral replication propagates in the host, it is expected to induce massive production of inflammatory cytokines from the host defense system, of which phenomena known as a cytokine storm (29). As montelukast showed therapeutic efficacy as a direct-acting antiviral agent against DV infection by increasing the survival rate in a mouse model, we further investigated whether the compound could reduce the immune response related to DV infection. The administration of montelukast decreased the level of representative inflammatory cytokines including tumor necrosis factor (TNF), interleukin-6 (IL-6), and IFN- γ as measured on the day when the virus titer in plasma was estimated to be at the highest level (Fig. 4D). In addition, complete blood count analysis was performed, in which the overall change in the blood components induced by the DV infection was attenuated, such as decrease of hematocrit at severe infection, increase of neutrophils and lymphocytes, and decrease of platelets (fig. S6) (30).

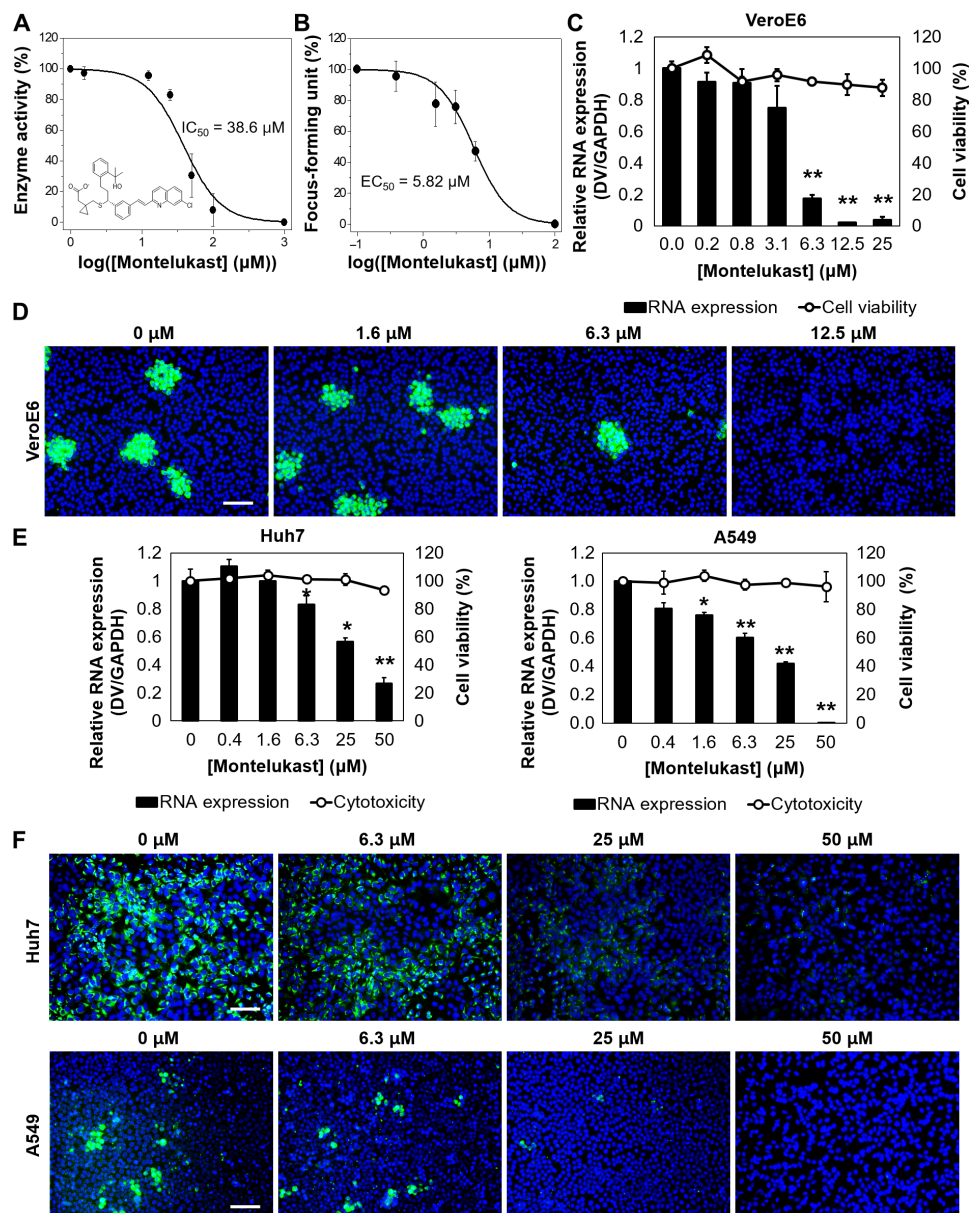


Fig. 3. The hit compound, montelukast, shows an in vitro antiviral therapeutic effect against the DV2-infected cells. (A) Chemical structure of the hit compound, montelukast, and investigation of the concentration-dependent enzyme inhibitory effect against DV RdRp via RANGO assay. The dose-responsive sigmoidal curve with the calculated IC₅₀ value is shown. Bars indicate means ± SEM from four individual groups for each concentration ($n = 4$). (B) Investigation of concentration-dependent antiviral effect on the hit compound via in vitro viral FFA on VeroE6 cells. The dose-responsive sigmoidal curve with the calculated EC₅₀ value is shown. Bars indicate means ± SEM from three individual groups for each concentration ($n = 3$). (C) Corresponding images of the viral focus of those treated with the representative concentration of the hit compound. Bars indicate means ± SEM from three independent groups for each concentration ($n = 3$). (D) Semiquantitative RNA expression analysis on the concentration-dependent antiviral effect of the hit compound and the corresponding cytotoxicity. (E) Relative viral RNA expression analysis of the concentration-dependent antiviral effect of the hit compound on the virus-infected human cells. Bars indicate means ± SEM from three individual groups for each concentration ($n = 3$). (F) Investigation of concentration-dependent antiviral effect on the hit compound via immunocytochemistry on human liver (Huh7) and lung carcinoma (A549) cells. To determine the significance of the data, Student's t test was performed to derive P value. * or ** indicates a significance in the change compared to the vehicle (PBS) control; * $P \leq 0.05$ and ** $P \leq 0.01$.

In addition to the investigation of the systemic antiviral effect of montelukast, the specific, local therapeutic impact on the peripheral organs was also examined. Five representative organs (spleen, liver, lung, small intestine, and large intestine) were collected and analyzed to estimate the changes in viral load and tissue morphology. As shown in Fig. 3E, administration of montelukast significantly reduced

the virus RNA concentration in each organ and in whole blood as well, although the degree of the viral RNA concentration decrease is different from organ to organ. To determine whether the change in viral RNA concentration in each organ is sufficient to relieve the infectious symptoms, the representative histopathology study on liver was performed, of which the unique symptoms of viral infection are

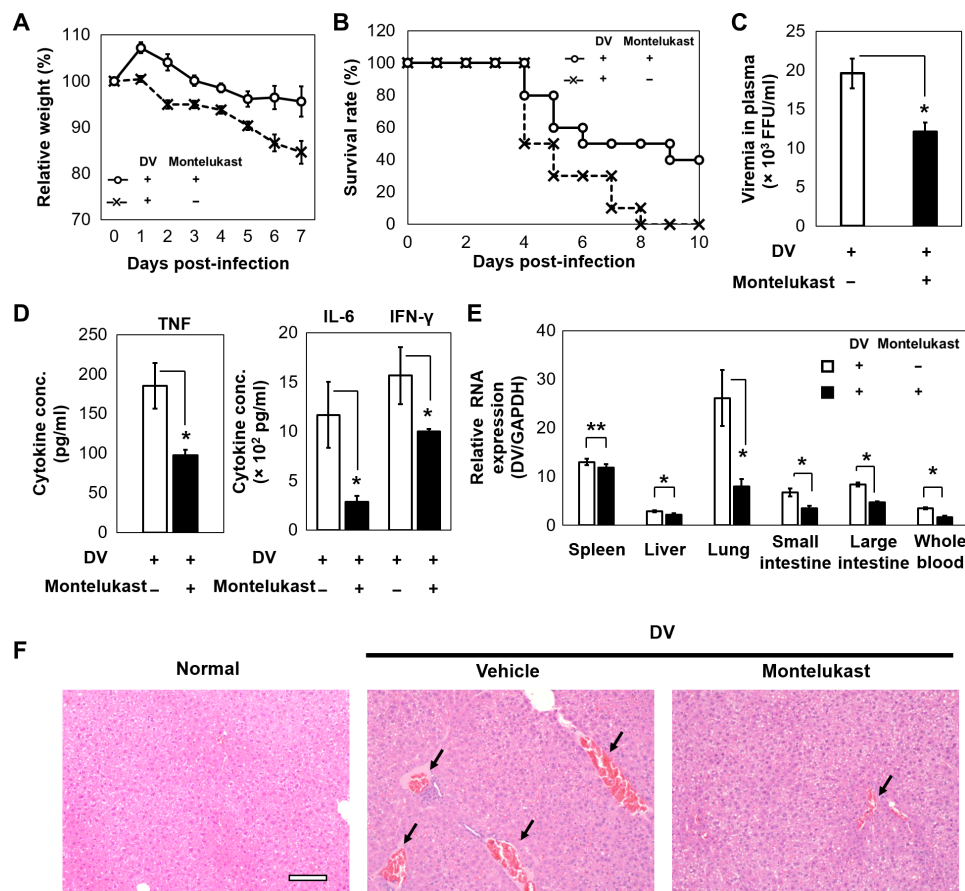


Fig. 4. Montelukast shows in vivo antiviral therapeutic efficacy against the DV2-infected mouse model. Montelukast (10 mg/kg) was treated once a day to DV2-infected AG129 mice (10 to 12 weeks old, $n = 10$ unless stated otherwise). **(A)** Relative weight change after the DV2 infection followed by the administration of vehicle or montelukast. The analysis includes data obtained up to day 7, as the number of mice in the negative control group (vehicle-treated) fell below 2 (which was insufficient to derive SD) on day 8. Bars indicate means $\pm$ SEM. **(B)** Survival rate analysis of DV-infected AG129 mice after daily intraperitoneal administration of montelukast. Statistical significance was estimated by log-rank test ($P = 0.023$). **(C)** Relative peak viremia reduction analysis in plasma, performed at day 3 after infection. **(D)** Cytokine analysis against inflammatory cytokines (TNF, IL-6, and IFN- γ). **(E)** Relative DV RNA expression level analysis was performed with each representative tissue sample (spleen, liver, lung, small intestine, large intestine, and whole blood). The overall semiquantification of RNA expression was normalized by endogenous control (GAPDH) and compared against each other. Bars indicate means $\pm$ SEM from six independent mice ($n = 6$). To determine the significance of the data, Student's t test was performed to derive P value. * or ** indicates a significant change compared to the vehicle (PBS) control; * $P \leq 0.05$ and ** $P \leq 0.01$. **(F)** Pathology analysis data. Hematoxylin and eosin-stained images of liver section are shown. Scale bar, 100 μ m.

well known (Fig. 4F) (31, 32). Several pathological features of the infection specific to liver are cell integrity loss, enlargement of the sinusoidal space, and the escalation of focal necrosis. It is generally known that the overall features of the cytopathic effect by the viral infection would eventually lead to the generation of the focal necrosis and apoptosis, resulting in the sinusoidal and lobular collapse to induce liver malfunctioning. It turned out that the administration of montelukast supported the notable maintenance of the cell integrity or the sinusoidal space, which resulted in the sufficient density of hepatocytes similar to that of the uninfected liver tissue. In addition, focal necrosis (labeled in black arrows) was also diminished by size and area in the liver tissue. Other organ-specific changes occur in spleen, of which size and weight increase upon virus infection (known as splenomegaly) (33). We found that administration of montelukast alleviated the development of splenomegaly compared to that of the untreated group (fig. S7). Collectively, the data indicate that the inhibition of DV replication by montelukast could significantly relieve various symptoms of DV infection and

achieve the comprehensive, systemic antiviral therapeutic effect in vivo.

DISCUSSION

Because of the tedious and laborious procedure and the complex handling required for enzyme-based parallel screening, our research was initiated with the aim of developing a facile platform to enhance the convenience and efficacy of antiviral agent discovery. To do so, we first devised the strategy to adopt the advantages of the GO-based biosensor to convert the enzyme activity into quantitatively analyzable fluorescence intensity. Next, we used the detailed protocol of RANGO to maintain its analytic validity when applied to the high-throughput screening. Because of the chemical stability of GO in conventional room conditions, this approach provides not only convenient storage and handling but also reduction of the experimental burden when operating RANGO. Z' -factor derivation and model inhibitor analysis based on our system proved its reproducibility

and reliability as a tool for the high-throughput screening of RdRp inhibitors. As a demonstration of the practical application of RANGO, we performed a multiwell-based, high-throughput chemical screening to identify a novel direct-acting antiviral agent against DV from the FDA-approved small-molecule library.

Categorized as an arbovirus, the generally known mechanism for DV infection starts from the mosquito bite and invasion of the virus into the bloodstream of the host, followed by infection of the epidermal tissues, tissue-residing immune cells, and lymph nodes, then expansion to systemic infection of peripheral organs (34). It has been reported that DV shows tissue tropism after the infection of the host, including bone marrow, lymph nodes, spleen, liver, and lungs (35). To prove that the RdRp inhibitor we identified could eventually become an anti-DV drug, it is necessary to determine whether the drug candidate is capable of practical viral reduction in at least one of these tissues stated above. We used various approaches involving a series of cell and animal-based models to evaluate the antiviral efficacy of the RdRp inhibitor selected from the RANGO-based screening. In addition to the monkey kidney cell model, we conducted additional cell experiments with the representative human cell models as well. It was necessary to examine whether the RdRp inhibitor candidate we found could show antiviral effects in human tissue. After the cell-based validation, we then conducted an animal-based study to eventually investigate the capability of the drug candidate to block the systemic spread-out of the viral infection. We managed to consider as many factors as possible to derive the conclusion, including the external symptoms (weight loss and survival rate), viral reduction in whole blood and each representative peripheral tissues, the relief of tissue-specific symptoms, cytokine level, and changes in the blood cells. Throughout the series of examinations, we concluded that the very compound, montelukast, showed significant potential as a direct-acting antiviral drug candidate.

Previously, montelukast has been known as a leukotriene receptor antagonist (36) and it is approved by the FDA for oral administration in treatment of chronic asthma. Related to viral infection, one previous report only showed that montelukast might possibly induce the partial reduction of vascular leakage occurring in by severe viral infection due to blocking mast cell-derived factor (37). However, no evidence was shown to claim that the compound could inhibit virus replication in any way, and the proof of concept was limited to the effect of the compound on the immune system as a leukotriene receptor antagonist. In the present study, we performed RANGO-based massive screening and identified montelukast as a highly potent DV RdRp inhibitor. We further performed a series of experiments to reveal the clear pieces of evidence supporting the role of montelukast as an antiviral agent. On the basis of the *in vitro* and *in vivo* data, we propose that montelukast could be adopted as a direct-acting antiviral drug targeting the DV RdRp. As far as we know, this mechanism of action (MoA) has not been suggested in previous studies regarding the antiviral effect of the compound. From the viewpoint of antiviral drugs, montelukast may enjoy its high efficacy as an anti-DV drug by dual MoAs as (i) a direct-acting antiviral agent by inhibiting DV RdRp and, therefore, inhibiting viral replication to directly reduce viral titer in the body and (ii) a leukotriene receptor antagonist to reduce the vascular leakage-related symptoms as well.

One of the biggest difficulties of antiviral drug development comes from rapid evolution of the target virus. The genetic insta-

bility of RNA virus leads to the continuous generation of variants alongside dispersal from one host to another. Future development of antiviral drugs, therefore, should be in a swift and strategic manner, with limited resources (i.e., time, labor, and money). Development of a drug screening platform, validated for its performance and efficiency, is thus an urgent issue. In terms of the drug screening system, the overall data indicate that our RANGO system is capable of rapid and reliable evaluation of the potent direct-acting antiviral drug candidates in a high-throughput manner, which is the superior feature compared to those of the conventional gel-based methods. Yet, regarding that our current system is based on the laboratory scale, the maximum number of samples in a single analysis is highly dependent on the experimental apparatus (e.g., in case of using 96-multiwell plates, a single analysis is capable of the maximum of ~80 samples, which takes approximately 5 to 10 min for the quantitative analysis). Thus, future investigations for the improvement of RANGO may consider strategies for application to massive, industrial-scale analysis to further increase efficacy of screening. One of the approaches may include the adoption of an automation process. Also, in terms of the validity of the repurposed drug candidate, our current results from the animal-based studies indicate the antiviral effect of montelukast when administered by intraperitoneal injection method in a single dose/day. Although montelukast is known to be treatable both intraperitoneally and orally, it had been widely transcribed orally for humans, which is accepted as a more convenient treatment route. Thus, further investigation is required for this drug to confirm both its antiviral effect via oral delivery and the optimized dose condition in clinical trials. Hopefully, as it is generally accepted that the systemic drug absorbance is greater by oral delivery than that of the intraperitoneal, the antiviral effect featuring the oral treatment of montelukast is expected to be positive.

In summary, we have developed the RANGO system, a cost-effective, simple, and robust RdRp activity analysis technology for the discovery of viral RdRp inhibitors. Its application to high-throughput screening of the FDA-approved drug library enabled the rapid selection of potent RdRp-specific inhibitors. In a series of studies carried out *in vitro* and *in vivo*, we identified a highly potent compound, montelukast, as a direct-acting antiviral drug candidate by inhibiting DV RdRp activity and, subsequently, DV replication mediated by the enzyme. On the basis of the significance of the preliminary data, we suggest that this small molecule selected by RANGO has great potential to be further investigated for practical use in combatting DV-associated diseases. More generally, we propose that our system could accelerate identification of potent direct-acting antiviral drug candidates at the initial stage of the drug discovery process.

MATERIALS AND METHODS

Cloning, expression, and purification of the RdRp recombinant protein

The coding sequence of the full-length NS5 protein from DV serotype 2 (DENV-2) was synthesized by Integrated DNA Technologies (USA). The DENV-2 NS5 sequence was fused to the coding sequence of the N-terminal 10 His-tagged superfolder green fluorescent protein (sfGFP) and inserted into the pX vector (38, 39). The expression plasmid was amplified in the *Escherichia coli* strain JM109, and the purified plasmids were transfected into human embryonic kidney

(HEK) 293E cells. The transfected cells were harvested 4 days after transfection and resuspended in the prepared buffer supplemented with ribonuclease A, staphylococcal nuclease (40), CaCl_2 , phenylmethylsulfonyl fluoride, and protease inhibitor cocktail. The resuspended cells were lysed by sonication. The supernatant was collected, and the desired protein was primarily purified by Ni-nitrilotriacetic acid (NTA) column. The eluted proteins were further purified using a HiLoad 16/600 Superdex 200 pg column (GE Healthcare, USA). The peak fractions were pooled and concentrated to approximately 1 mg/ml. The concentrated proteins were flash-frozen in liquid nitrogen and stored at -80°C before using in inhibitor screening assay.

Determination of the RdRp recombinant protein enzyme activity

The enzyme activity of the DV RdRp recombinant protein was determined by gel electrophoresis-based RNA analysis. To prepare DV serotype 2 RdRp substrate, ssRNA labeled with Cy5 was prepared (5'-Cy5-UUUUUUUUUUUUACUAACAACU-3'; Bioneer, Korea) in TE (Tris-EDTA) buffer solution. The DV2 RdRp enzyme (3.5 pmol) was mixed with 3 pmol of RNA substrate, 0.1 mM ATP, and 0.5 mM CTP/UTP/GTP in the 60- μl RdRp buffer (50 mM Hepes, 10 mM NaCl in pH 7.5) and incubated for 45 min at 37°C . Each sample was transferred to 20% native polyacrylamide gel electrophoresis (PAGE) gel, and electrophoresis was performed at 100 V for 20 to 30 min. Gels were stained with SYBR gold staining solution (Thermo Fisher Scientific, USA) and then analyzed using ChemiDoc (Bio-Rad, USA). The size of the resulting gel bands was determined compared to the small interfering RNA ladder marker (Takara Bio, Japan).

Synthesis of GO

GO was made according to the modified Hummers' method. Graphite, sodium nitrate, and sulfuric acid were mixed in round-bottom flask and vigorous stirring in an ice bath. After stabilization, we slowly added KMnO_4 . The flask was transferred to an oil bath and heated at 35°C for 1 hour. Distilled water (40 ml) was slowly dropped in the round flask in an ice bath. Then, the flask was placed in an oil bath at 95°C for 30 min. Reactive substances remaining in the flask were quenched by adding 5 ml of H_2O_2 , and the GO solution was purified by filtration using Buchner funnel. The purified GO was lyophilized and finally dispersed in distilled water to a concentration of 1 mg/ml.

Characterization of GO

The size and morphology of GOs were characterized with an atomic force microscope (NX10, Park Systems, Korea). An ultraviolet-visible (UV-Vis) spectrophotometer (Lambda 465, PerkinElmer, USA) was used to obtain UV-Vis spectra. Zeta potential and dynamic light scattering analysis were performed using Zetasizer Nano S (Malvern Instruments, UK). Raman spectra were measured using LabRAM HR UV-vis-NIR (Horiba Jobin Yvon, France) using an Ar ion continuous wave laser (514.5 nm) as an excitation source focused through a BXFM confocal microscope with an objective lens (50 $\times$, numerical aperture = 0.50). The measurement of Fourier transform infrared (FT-IR) spectra was performed with EQUINOX55 (Bruker, Germany).

Development of RNA nano-GO-based viral RdRp activity assay (RANGO)

The experimental procedures were performed in 96-well, black-bottom microplate. The reaction mixture for measuring RdRp ac-

tivity was prepared by mixing 3 pmol of RNA substrate, 3.5 pmol of DV2 RdRp and 0.1 mM ATP, and 0.5 mM CTP/UTP/GTP in the RdRp buffer (50 mM Hepes, 10 mM NaCl in pH 7.5) in 60 μl and incubated for 45 min at 37°C . NGO solution was prepared at 2.5 $\mu\text{g}/\text{ml}$ in distilled water right before use from a stock solution (1 mg/ml). After the incubation, NGO was added to each sample at a final concentration of 1.5 $\mu\text{g}/\text{ml}$. Reactants were transferred to the microplate. The eventual RdRp activity was measured by monitoring the corresponding fluorescence intensity change at excitation (Ex)/emission (Em) = 650 nm/670 nm with a Synergy MX fluorometer (BioTek, UK).

Evaluation of RANGO as the RdRp inhibitor assay platform

The experimental procedures were performed in 96-well, black-bottom microplate. For evaluation of the RANGO system as the RdRp inhibitor assay, the systemic performance was validated with a model RdRp inhibitor, ATA. Before the experiment, the compound solution was prepared in dimethyl sulfoxide (DMSO) in dark tube. ATA solution was added to the RdRp activity-induced buffer solution (with RdRp and RNA substrate) in serially diluted concentration from 0 to 300 μM . After 45 min of incubation at 37°C , NGO was added to each well at a final concentration of 1.5 $\mu\text{g}/\text{ml}$. Right after the addition of NGO, the fluorescence intensity was measured at Ex/Em = 650 nm/670 nm. Relative RdRp activity correlative to the difference in the fluorescence signal was plotted versus the treated inhibitor concentration, and the IC_{50} was calculated according to the logistic curve-fit method applied with OriginPro 8 (OriginLab, USA).

Z'-factor determination of RANGO

The experiment was conducted in 96-well plate scale. For the positive control, RdRp activity was induced by the addition of 3 pmol of RNA substrate, 3.5 pmol of DV serotype 2 RdRp and 0.1 mM ATP, and 0.5 mM CTP/UTP/GTP in the RdRp buffer (60 μl). For the negative control, the same volume of nuclease-free water (NFW) was added instead of NTP solution. After 45 min of incubation at 37°C , NGO was added to each well at a final concentration of 1.5 $\mu\text{g}/\text{ml}$. Right after the addition of NGO, the fluorescence intensity was measured at Ex/Em = 650 nm/670 nm ($n = 20$). The Z' factor was derived according to the equation as follows

$$Z' = 1 - \frac{(3\sigma_{c+} + 3\sigma_{c-})}{|\mu_{c+} - \mu_{c-}|}$$

where σ_{c+} is the SD of the positive control, σ_{c-} is the SD of the negative control, μ_{c+} is the average of the positive control, and μ_{c-} is the average of the negative control.

Screening performance test of RANGO

Preliminary screening test was performed against 80 random chemical compounds from the DIVERSet chemical library (ChemBridge Chemicals, USA). The chemicals were examined with either the conventional gel-based RNA assay or RANGO. Before the test, each chemical solution was diluted to 100 mM in DMSO. The solutions for each assay were prepared separately in a 96-well, black-bottom plate. Each well consisted of 3 pmol of RNA substrate, 3.5 pmol of DV serotype 2 RdRp and 0.1 mM ATP, 0.5 mM CTP/UTP/GTP in the RdRp buffer (60 μl), and 200 μM of library compound. For negative control, NTP was replaced with NFW, without any compound. The plates were incubated for 45 min at 37°C . For gel-based assay, each

analyte was transferred to the 20% native PAGE gel, and electrophoresis was performed in 100 V for 20 to 30 min. For RANGO-based assay, NGO was added directly to each well at a final concentration of 1.5 $\mu\text{g/ml}$. The fluorescence intensity was measured immediately after the addition of NGO, at Ex/Em = 650 nm/670 nm.

RANGO-based, high-throughput RdRp inhibitor screening

High-throughput chemical library screening was performed against 2400 candidate molecules from the FDA-approved chemical library (Selleckchem, USA) with the incorporation of RANGO system under the optimized condition as described above. Each chemical was added to the RdRp activity-induced buffer solution at a final concentration of 200 μM . For negative control, NTP was replaced with NFW. After 45 min of incubation at 37°C, NGO was added to each well at a final concentration of 1.5 $\mu\text{g/ml}$. Right after the addition of NGO, the fluorescence intensity was measured at Ex/Em = 650 nm/670 nm.

Quantitative enzyme inhibition analysis of the RdRp inhibitor candidate

For evaluation of the RdRp inhibition property of montelukast with the RANGO system, the compound solution was added to the RdRp activity-induced buffer solution in serially diluted concentration from 0 to 1000 μM . After 45 min of incubation at 37°C, NGO was added to each well at a final concentration of 1.5 $\mu\text{g/ml}$. Right after the addition of NGO, the fluorescence intensity was measured at Ex/Em = 650 nm/670 nm. Relative RdRp activity correlative to the difference in the fluorescence signal was plotted versus the treated drug concentration, and the IC_{50} was calculated according to the logistic curve-fit method applied with OriginPro 8 (OriginLab, USA).

Virus and cell culture

DV serotype 2 (strain KUMC-29) was provided from Korea Bank for Pathogenic Viruses at Korea University, Seoul, Korea. Monkey kidney cell line (VeroE6) was provided by K. Ahn from the Department of Biological Sciences, Seoul National University. Liver carcinoma cell line (Huh7) and lung carcinoma cell line (A549) were purchased from American Type Culture Collection Inc. (USA). The virus stock propagation and expansion for the concentration proceeded in VeroE6 cells. After harvest, the viruses were concentrated by ultracentrifugation at 36,000 rpm, 4°C for 3 hours. The concentrated titer was quantified by FFA. Virus titer was shown as FFU per milliliter. VeroE6 and Huh7 cell lines were cultured with complete Dulbecco's modified Eagle's medium in 5% CO_2 , 37°C. A549 cell line was cultured with complete RPMI medium (RPMI with 10% fetal bovine serum, 1% penicillin/streptomycin) in 5% CO_2 , 37°C.

Cell viability test

The cholecystokinin-8 (CCK-8) assay was performed according to the manufacturer's instruction. Before the experiment, VeroE6, Huh7, and A549 cells were seeded in densities of 10.5×10^4 , 7.0×10^4 , and 7.0×10^4 cells/ cm^2 , respectively. After 24 hours of incubation in 5% CO_2 , 37°C, montelukast was treated in serially diluted concentration for 48 hours. CCK-8 reagent was treated at concentrations of 10% (v/v) to each group and incubated for 1 to 4 hours in the conventional cell culture condition. The quantitative analysis of the change in colorimetric intensity was performed by measuring the optical density of formazan salt at 450 nm with a microplate reader (Molecular Devices Inc., USA). The experiment was carried out in triplicate, of which data were shown as means $\pm$ SEM.

In vitro virus infection test

Before the experiment, VeroE6, Huh7, and A549 cells were seeded in densities of 10.5×10^4 , 7.0×10^4 , and 7.0×10^4 cells/ cm^2 , respectively. After 24 hours of incubation under 5% CO_2 , 37°C, DV serotype 2 was inoculated to each cell culture by multiplicity of infection of 0.1, 0.5, and 2.5, respectively, under serum-free culture media for 2 hours. The culture plates were gently rocked every 30 min for even distribution of the virus to the cells. Montelukast solutions were prepared with a serially diluted concentration in each complete culture medium containing 0.75% methylcellulose. After the incubation, virus medium was removed and the cells were washed with sterilized PBS once, followed by the treatment of the chemical solutions. Cells were incubated under the culture chamber for 48 hours. After the incubation, each group was prepared for the FFA and relative viral RNA expression analysis. For FFA, chemical solutions were removed and the cells were washed with sterilized PBS, followed by fixation with 4% paraformaldehyde. For viral RNA expression analysis, cells were treated with TRIzol after the washing process and stored at -70°C for further analysis.

Focus-forming assay

FFA was performed against the NS1 protein of DV serotype 2. Cells fixed with 4% paraformaldehyde were rinsed with PBS for three times. The blocking solution was treated for 1 hour at room temperature. After washing with PBS, primary antibody (mouse anti-flavivirus NS1; 1:2000) was treated to each group for 2 hours at room temperature or overnight at 4°C. After washing with PBS, secondary antibody [anti-mouse immunoglobulin G (IgG)–fluorescein isothiocyanate (FITC); 1:500] was applied to each group for 1 hour at room temperature. After rinsing, PBS containing Hoechst 33342 (10 $\mu\text{g/ml}$) was treated to each group for nuclear staining. Viral FFUs stained in fluorescence were observed and analyzed with a microscope (Olympus, Japan).

Animal experiments

AG129 mice (129/Sv IFN- α/β , IFN- γ receptor-deficient) were purchased from Marshall BioResources (UK). All experimental procedures were preapproved by Institutional Animal Care and Use Committee of Seoul National University (Korea) and Chonbuk National University (Korea) and were performed according to the guidelines of the recommendations from Association for Assessment and Accreditation of Laboratory Animal Care. As the DV is epidemic by mosquitoes, the cages with filter cover were used to avoid any unexpected contact and contamination.

Experimental groups for in vivo virus infection test

For determination of the appropriate virus titer for infection, AG129 mice (10 to 12 weeks) were inoculated by intraperitoneal injection with various viral titer: 10^6 , 5×10^6 , and 10^7 FFU. For evaluation of the antiviral properties of montelukast, AG129 mice (10 to 12 weeks) were intraperitoneally injected with DV (10^7 FFU), followed by intraperitoneal administration of either 200 μl of montelukast (10 mg/kg per day) or PBS. Drug treatment was performed once a day. Negative control (uninfected group) was administered with 300 μl of PBS instead of virus. For drug control group, 200 μl of montelukast (10 mg/kg per day) was administered solely to the noninfected AG129 mice.

Mouse weight loss and survival rate analysis

Weight loss and mortality state were monitored daily. In the survival analysis, euthanized mice exhibiting severe disease-associated

symptoms or rapid weight loss were counted as the moribund state at the moment of the exhibition. Each weight was analyzed in relative percentage against that of day 0.

Complete blood count, viremia analysis, whole-blood RNA expression analysis, and cytokine bead assay

At day 3 after infection, blood was collected from each mouse and transferred into EDTA-coated anticoagulant tubes. After gentle mix by rocking for 10 min, 20 μ l of each sample was analyzed for complete blood counts with Hemavet 950FS (Drew Scientific, USA). The measured value of each factor was semiquantitatively analyzed against that of the uninfected control. For analysis of viremia and expression change in cytokines, plasma was separated by centrifugation at 8000g, 4°C for 15 min. Virus titer in the plasma (viremia) and viral RNA expression in whole blood was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). To examine change in the expression of inflammatory cytokines (TNF, IL-6, and IFN- γ), cytokine bead assay was performed. All samples were analyzed according to the manufacturer's instruction (BD Biosciences, USA).

Mouse necropsy

At day 3 after infection, mice were sacrificed from each group ($n = 6$). Organ samples (spleen, liver, lung, small intestine, and large intestine) were collected and prepared for the virus RNA expression analysis and histopathology analysis. Spleens were weighed to determine splenomegaly. For the virus RNA expression analysis, half of the spleen, liver, and lung were homogenized and treated with TRIzol reagent and stored at -70°C for further analysis. The same procedures were applied to the whole samples of the small intestine and large intestine. The other half of the spleen, liver, and lung were submerged to 4% paraformaldehyde for fixation and stored at 4°C.

Relative viral RNA expression level analysis

For the investigation of viral RNA expression change in the collected specimens, RNA was isolated from each cell- and animal-based sample treated with TRIzol reagent, followed by complementary DNA synthesis aided by MLVRT reverse transcriptase according to the instruction manual. For target gene amplification, each primer was designed in consideration of GC (guanine-cytosine) content less than 50% and overlapping between two exons of the target genes with the expected amplicon size of ~ 100 base pairs. Primer sequence was confirmed as shown in table S1. qRT-PCR was conducted to quantify viral RNA. Each reaction was conducted in a 20- μ l volume, with SYBR Green Master Mix. QuantStudio3 (Applied Biosystems Inc., USA) was used in this study in a two-step amplification process. For analysis of viremia, a linear correlative standard curve was established between the virus titer (FFU per milliliter) and the corresponding threshold cycle (C_T) value. The virus in plasma was calculated according to the equation of the standard curve. A standard curve was derived against a serially diluted viral titer. For cell-based groups and mice organ samples (including whole blood), semiquantitative viral RNA was analyzed against that of the endogenous control, GAPDH.

Pathology analysis

Organ samples fixed in 4% paraformaldehyde went through a series of procedures as follows: paraffin embedding, section, and stain with hematoxylin and eosin at the pathology core facility in Center for Medical Innovation, Seoul National University Hospital, Korea.

Statistical analysis

Data plots with concentration-dependent assays were analyzed using OriginPro 8. Briefly, logarithmic sigmoidal curves were fitted according to each dataset. Error bars indicate $\pm$ SEM unless stated otherwise. Statistical significance of the in vitro and in vivo antiviral efficacy analysis was evaluated by a two-tailed t test. Statistical significance of the survival rate was analyzed by log-rank test with R (R Foundation for Statistical Computing, Austria).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/6/22/eaaz8201/DC1>

[View/request a protocol for this paper from Bio-protocol.](#)

REFERENCES AND NOTES

1. J. S. Mackenzie, D. J. Gubler, L. R. Petersen, Emerging flaviviruses: The spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. *Nat. Med.* **10**, S98–S109 (2004).
2. A. P. S. Rathore, A. L. St. John, Immune responses to dengue virus in the skin. *Open Biol.* **8**, 180087 (2018).
3. H. K. Janai, M. I. Marks, M. Zaleska, H. R. Stutman, Ribavirin: Adverse drug reactions, 1986 to 1988. *Pediatr. Infect. Dis. J.* **9**, 209–211 (1990).
4. E. De Clercq, G. Li, Approved antiviral drugs over the past 50 years. *Clin. Microbiol. Rev.* **29**, 695–747 (2016).
5. J. G. H. Low, E. E. Ooi, S. G. Vasudevan, Current status of dengue therapeutics research and development. *J. Infect. Dis.* **215**, S96–S102 (2017).
6. T. F. Baumert, T. Berg, J. K. Lim, D. R. Nelson, Status of direct-acting antiviral therapy for hepatitis C virus infection and remaining challenges. *Gastroenterology* **156**, 431–445 (2019).
7. J. G. Low, C. Sung, L. Wijaya, Y. Wei, A. P. S. Rathore, S. Watanabe, B. H. Tan, L. Toh, L. T. Chua, Y. Hou, A. Chow, S. Howe, W. K. Chan, K. H. Tan, J. S. Chung, B. P. Cherng, D. C. Lye, P. A. Tambayah, L. C. Ng, J. Connolly, M. L. Hibberd, Y. S. Leo, Y. B. Cheung, E. E. Ooi, S. G. Vasudevan, Efficacy and safety of celgosivir in patients with dengue fever (CELADEN): A phase 1b, randomised, double-blind, placebo-controlled, proof-of-concept trial. *Lancet Infect. Dis.* **14**, 706–715 (2014).
8. N. M. Nguyen, C. N. Tran, L. K. Phung, K. T. Duong, H. le Anh Huynh, J. Farrar, Q. T. H. Nguyen, H. T. Tran, C. V. V. Nguyen, L. Merson, L. T. Hoang, M. L. Hibberd, P. P. K. Aw, A. Wilm, N. Nagarajan, D. T. Nguyen, M. P. Pham, T. T. Nguyen, H. Javanbakht, K. Klumpp, J. Hammond, R. Petric, M. Wolbers, C. T. Nguyen, C. P. Simmons, A randomized, double-blind placebo controlled trial of balapiravir, a polymerase inhibitor, in adult dengue patients. *J. Infect. Dis.* **207**, 1442–1450 (2013).
9. R. Dulbecco, Production of plaques in monolayer tissue cultures by single particles of an animal virus. *Proc. Natl. Acad. Sci. U.S.A.* **38**, 747–752 (1952).
10. F. Guo, X. Zhao, T. Gill, Y. Zhou, M. Campagna, L. Wang, F. Liu, P. Zhang, L. DiPaolo, Y. Du, X. Xu, D. Jiang, L. Wei, A. Cuconati, T. M. Block, J.-T. Guo, J. Chang, An interferon-beta promoter reporter assay for high throughput identification of compounds against multiple RNA viruses. *Antiviral Res.* **107**, 56–65 (2014).
11. M. Qing, W. Liu, Z. Yuan, F. Gu, P. Y. Shi, A high-throughput assay using dengue-1 virus-like particles for drug discovery. *Antiviral Res.* **86**, 163–171 (2010).
12. C. M. Byrd, D. W. Grosenbach, A. Berhanu, D. Dai, K. F. Jones, K. B. Cardwell, C. Schneider, G. Yang, S. Tyavanagimatt, C. Harver, K. A. Wineinger, J. Page, E. Stavale, M. A. Stone, K. P. Fuller, C. Lovejoy, J. M. Leeds, D. E. Hrubby, R. Jordan, Novel benzoxazole inhibitor of dengue virus replication that targets the NS3 helicase. *Antimicrob. Agents Chemother.* **57**, 1902–1912 (2013).
13. C.-C. Yang, H.-S. Hu, R.-H. Wu, S.-H. Wu, S.-J. Lee, W.-T. Jiaang, J.-H. Chern, Z.-S. Huang, H.-N. Wu, C.-M. Chang, A. Yueh, A novel dengue virus inhibitor, BP13944, discovered by high-throughput screening with dengue virus replicon cells selects for resistance in the viral NS2B/NS3 protease. *Antimicrob. Agents Chemother.* **58**, 110–119 (2014).
14. G. Lu, P. Gong, A structural view of the RNA-dependent RNA polymerases from the *Flavivirus* genus. *Virus Res.* **234**, 34–43 (2017).
15. H. Shimizu, A. Saito, J. Mikuni, E. E. Nakayama, H. Koyama, T. Honma, M. Shirouzu, S.-i. Sekine, T. Shioda, Discovery of a small molecule inhibitor targeting dengue virus NS5 RNA-dependent RNA polymerase. *PLOS Negl. Trop. Dis.* **13**, e0007894 (2019).
16. Y. Zhu, S. Murali, W. Cai, X. Li, J. W. Suk, J. R. Potts, R. S. Ruoff, Graphene and graphene oxide: Synthesis, properties, and applications. *Adv. Mater.* **22**, 3906–3924 (2010).
17. C.-H. Lu, H.-H. Yang, C.-L. Zhu, X. Chen, G.-N. Chen, A graphene platform for sensing biomolecules. *Angew. Chem. Int. Ed. Engl.* **48**, 4785–4787 (2009).
18. J. Kim, S.-J. Park, D.-H. Min, Emerging approaches for graphene oxide biosensor. *Anal. Chem.* **89**, 232–248 (2017).

19. H. Jang, S.-R. Ryoo, M. J. Lee, S. W. Han, D.-H. Min, A new helicase assay based on graphene oxide for anti-viral drug development. *Mol. Cells* **35**, 269–273 (2013).
20. J. Lee, I.-S. Park, G. Park, K. Cho, H.-S. Park, D.-H. Min, A robust and quantitative assay platform for multiplexed, high throughput screening of protein kinase inhibitors. *Chem. Commun.* **52**, 12112–12115 (2016).
21. D. Li, W. Zhang, X. Yu, Z. Wang, Z. Su, G. Wei, When biomolecules meet graphene: From molecular level interactions to material design and applications. *Nanoscale* **8**, 19491–19509 (2016).
22. J.-H. Zhang, T. D. Y. Chung, K. R. Oldenburg, A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen.* **4**, 67–73 (1999).
23. Y. Chen, A. Bopda-Waffo, A. Basu, R. Krishnan, E. Silberstein, D. R. Taylor, T. T. Talele, P. Arora, N. Kaushik-Basu, Characterization of aurointricarboxylic acid as a potent hepatitis C virus replicase inhibitor. *Antivir. Chem. Chemother.* **20**, 19–36 (2009).
24. S. J. Balsitis, J. Coloma, G. Castro, A. Alava, D. Flores, J. H. McKerrow, P. R. Beatty, E. Harris, Tropism of dengue virus in mice and humans defined by viral nonstructural protein 3-specific immunostaining. *Am. J. Trop. Med. Hyg.* **80**, 416–424 (2009).
25. Y.-R. Lee, C.-Y. Su, N.-H. Chow, W.-W. Lai, H.-Y. Lei, C.-L. Chang, T.-Y. Chang, S.-H. Chen, Y.-S. Lin, T.-M. Yeh, H.-S. Liu, Dengue viruses can infect human primary lung epithelia as well as lung carcinoma cells, and can also induce the secretion of IL-6 and RANTES. *Virus Res.* **126**, 216–225 (2007).
26. W. Schul, W. Liu, H.-Y. Xu, M. Flamand, S. G. Vasudevan, A dengue fever viremia model in mice shows reduction in viral replication and suppression of the inflammatory response after treatment with antiviral drugs. *J. Infect. Dis.* **195**, 665–674 (2007).
27. K. L. Williams, S. Zompi, P. R. Beatty, E. Harris, A mouse model for studying dengue virus pathogenesis and immune response. *Ann. N. Y. Acad. Sci.* **1171**, E12–E23 (2009).
28. V. V. Sarathy, M. White, L. Li, S. R. Gorder, R. B. Pyles, G. A. Campbell, G. N. Milligan, N. Bourne, A. D. T. Barrett, A lethal murine infection model for dengue virus 3 in AG129 mice deficient in type I and II interferon receptors leads to systemic disease. *J. Virol.* **89**, 1254–1266 (2015).
29. A. L. Rothman, Immunity to dengue virus: A tale of original antigenic sin and tropical cytokine storms. *Nat. Rev. Immunol.* **11**, 532–543 (2011).
30. F. A. Bozza, O. G. Cruz, S. M. O. Zagne, E. L. Azeredo, R. M. R. Nogueira, E. F. Assis, P. T. Bozza, C. F. Kubelka, Multiplex cytokine profile from dengue patients: MIP-1 β and IFN- γ as predictive factors for severity. *BMC Infect. Dis.* **8**, 86 (2008).
31. S. Shrestha, K. L. Sharar, D. M. Prigozhin, P. R. Beatty, E. Harris, Murine model for dengue virus-induced lethal disease with increased vascular permeability. *J. Virol.* **80**, 10208–10217 (2006).
32. D. F. Barreto, C. M. Takiya, H. G. Schatzmayr, R. M. R. Nogueira, J. da Costa Farias-Filho, O. M. Barth, Histopathological and ultrastructural aspects of mice lungs experimentally infected with dengue virus serotype 2. *Mem. Inst. Oswaldo Cruz* **102**, 175–182 (2007).
33. T. F. Póvoa, A. M. B. Alves, C. A. B. Oliveira, G. J. Nuovo, V. L. A. Chagas, M. V. Paes, The pathology of severe dengue in multiple organs of human fatal cases: Histopathology, ultrastructure and virus replication. *PLOS ONE* **9**, e83386 (2014).
34. B. E. E. Martina, P. Koraka, A. D. M. E. Osterhaus, Dengue virus pathogenesis: An integrated view. *Clin. Microbiol. Rev.* **22**, 564–581 (2009).
35. J. L. Kyle, P. R. Beatty, E. Harris, Dengue virus infects macrophages and dendritic cells in a mouse model of infection. *J. Infect. Dis.* **195**, 1808–1817 (2007).
36. T. F. Reiss, P. Chervinsky, R. J. Dockhorn, S. Shingo, B. Seidenberg, T. B. Edwards; Montelukast Clinical Research Study Group, Montelukast, a once-daily leukotriene receptor antagonist, in the treatment of chronic asthma: A multicenter, randomized, double-blind trial. *Arch. Intern. Med.* **158**, 1213–1220 (1998).
37. A. L. St. John, A. P. S. Rathore, B. Raghavan, M.-L. Ng, S. N. Abraham, Contributions of mast cells and vasoactive products, leukotrienes and chymase, to dengue virus-induced vascular leakage. *eLife* **2**, e00481 (2013).
38. T. A. Nguyen, M. H. Jo, Y.-G. Choi, J. Park, S. C. Kwon, S. Hohng, V. N. Kim, J.-S. Woo, Functional anatomy of the human microprocessor. *Cell* **161**, 1374–1387 (2015).
39. G. Backliwal, M. Hildinger, S. Chenuet, S. Wulhfard, M. De Jesus, F. M. Wurm, Rational vector design and multi-pathway modulation of HEK 293E cells yield recombinant antibody titers exceeding 1 g/l by transient transfection under serum-free conditions. *Nucleic Acids Res.* **36**, e96 (2008).
40. P. Cuatrecasas, S. Fuchs, C. B. Anfinsen, Catalytic properties and specificity of the extracellular nuclease of *Staphylococcus aureus*. *J. Biol. Chem.* **242**, 1541–1547 (1967).

Acknowledgments

Funding: This work was supported by the Basic Science Research Program (2016R1E1A1A01941202) International S&T Cooperation Program (2014K1B1A1073716), Lemonex Inc. (LEMONEX-SN201709-3-12 and LEMONEX-SN201901-3-30), and the Research Center Program (IBS-R008-D1) of Institute for Basic Science (IBS) through the National Research Foundation of Korea (NRF). **Author contributions:** S.-J.P. designed and performed the RANGO-based high-throughput screening for RdRp inhibitor candidates. S.-J.P. and J.K. designed and performed the in vitro and in vivo experiments and data analysis. S.K. and H.S. carried out the experiments associated with the synthesis and characterization of NGO. H.J.C. and J.-S.W. performed the experiments associated with gene cloning, expression of recombinant RdRp protein, and its characterization. J.P. and Y.-S.J. managed the animal experiments and performed pilot in vivo experiments. C.W. and D.-H.M. conceived the hypotheses, performed the analysis and interpretations, and wrote the paper. **Competing interests:** C.W. and D.-H.M. are inventors of a pending patent related to this work filed by Lemonex Inc. (no. KR20190125943A). The remaining authors declare no competing interests. **Data and materials availability:** All data required to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 11 October 2019

Accepted 31 March 2020

Published 29 May 2020

10.1126/sciadv.aaz8201

Citation: S.-J. Park, J. Kim, S. Kang, H. J. Cha, H. Shin, J. Park, Y.-S. Jang, J.-S. Woo, C. Won, D.-H. Min, Discovery of direct-acting antiviral agents with a graphene-based fluorescent nanosensor. *Sci. Adv.* **6**, eaaz8201 (2020).

Discovery of direct-acting antiviral agents with a graphene-based fluorescent nanosensor

Se-Jin Park, Jungho Kim, Seounghun Kang, Hyung Jin Cha, Hojeong Shin, Jisang Park, Yong-Suk Jang, Jae-Sung Woo, Cheolhee Won and Dal-Hee Min

Sci Adv **6** (22), eaaz8201.
DOI: 10.1126/sciadv.aaz8201

ARTICLE TOOLS

<http://advances.sciencemag.org/content/6/22/eaaz8201>

SUPPLEMENTARY MATERIALS

<http://advances.sciencemag.org/content/suppl/2020/05/21/6.22.eaaz8201.DC1>

REFERENCES

This article cites 40 articles, 9 of which you can access for free
<http://advances.sciencemag.org/content/6/22/eaaz8201#BIBL>

PERMISSIONS

<http://www.sciencemag.org/help/reprints-and-permissions>

Use of this article is subject to the [Terms of Service](#)

Science Advances (ISSN 2375-2548) is published by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005. The title *Science Advances* is a registered trademark of AAAS.

Copyright © 2020 The Authors, some rights reserved; exclusive licensee American Association for the Advancement of Science. No claim to original U.S. Government Works. Distributed under a Creative Commons Attribution NonCommercial License 4.0 (CC BY-NC).