

Repurposing of Prochlorperazine for Use Against Dengue Virus Infection

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The increasing prevalence of dengue virus (DENV) infection presents serious disease and economic burdens in countries where dengue epidemics are occurring. Despite the clinical importance, no DENV vaccine or anti-DENV drug is available. In this study, we found that prochlorperazine (PCZ), a dopamine D2 receptor (D2R) antagonist approved to treat nausea, vomiting, and headache in humans has potent *in vitro* and *in vivo* antiviral activity against DENV infection. PCZ can block DENV infection by targeting viral binding and viral entry through D2R- and clathrin-associated mechanisms, respectively. Administration of PCZ immediately or 6 hours after DENV infection in a *Stat1*-deficient mouse model completely protected against or delayed lethality. Overall, PCZ showed a previously unknown antiviral effect against DENV infection, and D2R may play a role in the DENV life cycle. Prophylactic and/or therapeutic treatment with PCZ might reduce viral replication and relieve the clinical symptoms of patients with dengue.

Keywords. drug repurposing; dengue virus; prochlorperazine; clathrin-mediated endocytosis; dopamine D2 receptor.

Dengue virus (DENV) infection in humans can cause a wide spectrum of illnesses, ranging from mild dengue fever to severe dengue hemorrhagic fever and life-threatening dengue shock syndrome [1, 2]. The virus is transmitted by *Aedes* mosquitoes, and >40% of the world's population living in tropic and subtropic areas are at risk of DENV infection. The World Health Organization estimates a prevalence of 50–100 million cases of DENV infection annually; however, a recent study suggested 390 million DENV infections occur per year [3]. Although the mortality rate has been successfully controlled in recent years, recurrent dengue outbreaks and increased prevalence have significant socioeconomic impact in countries where dengue epidemics are occurring [4, 5].

DENV, belonging to the *Flaviviridae* family, is enveloped and contains a positive-sense RNA genome

encoding a polyprotein that is processed to 3 structural and 7 nonstructural proteins by cellular and viral proteases [6, 7]. DENV infection starts by binding to cell surface receptor(s) through the major structural envelope protein and then enters cells via clathrin-mediated endocytosis [8]. The internalized virions undergo acid-induced conformational changes and membrane fusion to release the viral genome. Translation of viral RNA produces proteins required for viral RNA replication through RNA-dependent RNA polymerization. The assembly of viral RNA and viral proteins generates the mature viral particle, which is then released through the cellular secretory pathway.

Vaccines and antiviral drugs are 2 major means to control viral diseases, but DENV vaccines and anti-DENV drugs are not yet available. The development of a DENV vaccine has been hampered in part because of the complexity of the 4 serotypes (DENV-1–4) and the potential involvement of antibody-dependent enhancement in severe dengue [1, 9]. Over the decades, many dengue vaccine candidates have been developed [9, 10], and a recombinant, live-attenuated tetravalent dengue vaccine was recently evaluated in a randomized, controlled phase 2b clinical trial [11]. Although the vaccine was well tolerated, the overall efficacy, 30.2%, was

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lower than expected. Thus, a safe and effective vaccine against all 4 DENV serotypes for human use may not be available in the near future. For antiviral development, both viral and cellular proteins essential for viral replication serve as potential targets. Some prospective candidates with promising antiviral effects against DENV infection both in vitro and in vivo have been reported [12–18]. However, the use of these novel compounds is constrained by uncharacterized drug pharmacokinetics, dosing, and safety information for humans. Consequently, the translational research bringing the laboratory findings to the bedside of patients with dengue is still challenging [19].

Research of clinically approved drugs for new indications, referred to as “drug repurposing,” can play a substantial role in accelerating the development of antiviral drugs. Prochlorperazine (PCZ) is clinically approved to treat headache, nausea, and vomiting, which are also common symptoms among patients with dengue [20]. PCZ is a dopamine D2 receptor (D2R) antagonist and belongs to the phenothiazine class of antipsychotic agents [21]. In a cell-based screening study, PCZ exhibited an antiviral effect against hepatitis C virus (HCV), also a *Flaviviridae* member, by targeting virus entry [22]. Members of *Flaviviridae* may share similarities in their life cycles and host–pathogen interactions [23, 24], and chlorpromazine, a compound with a structure similar to that of PCZ, inhibits clathrin-dependent endocytosis [25], which is important for the entry of *Flaviviridae* members such as DENV and HCV [25–28]. Thus, in this study, we aimed to examine the in vitro and in vivo antiviral potential of PCZ against DENV infection and elucidate the drug’s possible antiviral mechanisms.

MATERIALS AND METHODS

Cell-Based Antiviral Assay

The cell lines, virus strains, and drugs used in this study are described in the [Supplementary Materials](#). Cells in 6- or 12-well plates were adsorbed with DENV-2 at the indicated multiplicity of infection (MOI) for 2 hours with the indicated concentrations of PCZ, washed thoroughly to remove unbound viruses, then incubated for another 24–48 hours in the presence or absence of PCZ. The antiviral effect of PCZ was evaluated by immunofluorescence assay, immunoblotting, and plaque-forming assay as described in [Supplementary Data](#). The half maximal effective concentration (EC₅₀) was calculated by means of a published method [29].

Virus-Binding and Virus-Entry Assays

For the virus-binding assay, cells pretreated with the indicated doses of PCZ for 1 hour were adsorbed with DENV-2 for 2 hours at 4°C with rocking on a linear shaker in the presence or absence of PCZ. For virus entry assay, cells were adsorbed with DENV-2 for 2 hours at 4°C without PCZ. At 15 minutes before the end of virus adsorption, PCZ was added to the cell

cultures. After a gentle wash with cold phosphate-buffered saline (PBS), cells were shifted incubated at 37°C for 1 hour in the presence or absence of PCZ to allow virus entry. Virus binding and entry were evaluated by confocal microscopy and plaque-forming assay. Fluorescently labeled DENV-2 prepared as described in [Supplementary Materials](#) was used for confocal microscopy.

Virus-Binding Competition Assay

Cells were adsorbed with fluorescently labeled DENV-2 (MOI, 5) for 2 hours at 4°C with rabbit anti-D2R antibody (sc-91113; Santa Cruz Biotechnology) or rabbit immunoglobulin G (IgG) as a nonspecific antibody control. For confocal microscopy, cells were incubated with Hoechst for 15 minutes at 4°C before the end of virus adsorption. The D2R-specific ligand N-methylpiperone was also used in the competition assay. Cells were adsorbed with fluorescently labeled DENV-2 (MOI, 40) (for confocal microscopy) or DENV-2 (MOI, 50; for quantification by plaque-forming assay) for 30 minutes at 4°C with the indicated doses of N-methylpiperone. Virus binding was determined by confocal microscopy and plaque-forming assays.

Lentivirus Preparation and D2R Knockdown

The lentivirus vector pLKO.1, which carries a short hairpin RNA (shRNA) targeting the mouse D2R (5′-CCACTACAACACTATGCCAT-3′, TRCN0000220818) or LacZ (5′-TGTTTCGCATTATCCGAACCAT-3′, TRCN0000072223), was obtained from the Taiwan National RNAi Core Facility and cotransfected with pMD.G and pCMVΔR8.91 into HEK293T cells for lentivirus production and cell transduction for D2R knockdown, as described in the [Supplementary Materials](#). The rescue experiment was done in shD2R-N18 cells by transduction with lentivirus vector pLKO_AS3w expressing murine D2R_L (NM_010077) or D2R_S (M36831). Site-directed mutagenesis was used to introduce a wobble mutation without changing protein sequences, to generate shD2R-resistant D2R_L and D2R_S with the primer sequences 5′-AGGGAAGCCAGACAGGCCA CATTATAATTATTACGCTATGCTGCTCACCCCTCCTCAT-3′ (mutated sequences are underlined).

Animal Study

The mouse experiments were approved and performed in accordance with the guidelines of the Academia Sinica Institutional Animal Care and Use Committee. Groups of 5-week-old *Stat1*^{-/-} mice were challenged intraperitoneally with 5 × 10⁴ plaque-forming units (PFU)/mouse of DENV-2 New Guinea C serially passaged in mouse brain (NGC-N) strain and simultaneously injected with 30 μL of PBS intracranially into the right hemisphere of the brain [30, 31]. PCZ dimaleate was administered mainly by an oral route, and PCZ-Novamin was administered by parenteral methods ([Supplementary Materials](#)).

Statistical Analysis

Data are presented as mean \pm standard deviation and were compared by the Mann–Whitney *U* test. Statistical significance was set at *P* values of $< .05$ and $< .01$. A survival curve was descriptively analyzed by use of SigmaPlot v10.0 (Systat Software). Determination of the median survival time (T_{50}) and *P* values, by the log-rank test, involved use of Prism v5.0 (GraphPad Software). For immunoblotting, the band density was quantified by use of ImageJ software (National Institutes of Health).

RESULTS

PCZ Exhibits Antiviral Effects Against DENV-2 Infection

We first determined the noncytotoxic doses of a clinically used PCZ, prochlorperazine dimethanesulfonate (Novamin injection; PCZ-Novamin), with 3 different assays: the AlamarBlue Cell Viability assay, the XTT Cell Proliferation assay, and the LDH Cytotoxicity assay. PCZ (up to 30 μ M) had no significant effect on cell viability, cell proliferation, or cytotoxicity in several cell lines, such as human kidney HEK293T (Figure 1A), lung A549, microglia CHME3, and monocytic THP-1 cells and mouse neuroblastoma N18 cells (data not shown). Treatment with noncytotoxic PCZ significantly reduced DENV-2 viral protein expression and viral progeny production in a dose-dependent manner in HEK293T cells (Figure 1B–D), with an EC_{50} of 88 nM. The anti-DENV-2 activity of PCZ was also noted in CHME3 cells and THP-1 cells with DC-SIGN overexpression (Supplementary Figure 1A and 1B).

We further evaluated the antiviral potential of a PCZ solution prepared from PCZ dimaleate. Similarly, PCZ dimaleate dose-dependently blocked DENV-2 replication at noncytotoxic doses (Figure 1E and 1F), with an EC_{50} of 137 nM, slightly higher than that of PCZ-Novamin probably because of different compound solubilities. Thus, at noncytotoxic concentrations, PCZ showed antiviral effects against DENV-2 infection. Furthermore, PCZ may tackle early steps of viral infection, because 4 hours of treatment with PCZ-Novamin during viral adsorption dose-dependently reduced DENV-2 production (Supplementary Figure 1C). In addition, PCZ suppressed the infection of other flaviviruses, such as DENV-1 and Japanese encephalitis virus (Supplementary Figure 1D and 1E), so PCZ may have a broad antiviral potential against members of the *Flaviviridae* family.

PCZ Inhibits DENV-2 Entry Through a Clathrin-Associated Pathway

To determine the antiviral mechanism of PCZ, we investigated whether PCZ can block DENV entry. We performed a virus-entry assay by using fluorescently labeled DENV-2. Without PCZ treatment, after 1 hour of incubation at 37°C, many green signals of fluorescently labeled DENV-2 were seen in the solvent-treated cells by confocal microscopy; however, the

DENV-2 signals were greatly reduced in PCZ-treated cells (Figure 2A). The amount of internalized DENV-2 quantified by plaque-forming assay was also significantly reduced with PCZ in a dose-dependent manner (Figure 2B). Similar to treatment with chlorpromazine, which is structurally similar to PCZ and can disrupt clathrin distribution [25], 30-minute treatment with PCZ (20 and 30 μ M) affected clathrin distribution in mock- and DENV-2-infected HEK293T cells (Figure 2C). Furthermore, with PCZ treatment, DENV-2 colocalized with clathrin on the cell surface without entering the cells (Figure 2C). Thus, PCZ may affect clathrin distribution, which in turn blocks DENV entry through clathrin-mediated endocytosis.

PCZ Blocks DENV-2 Binding in a D2R-Dependent Manner

We then performed a virus-binding assay at 4°C for 2 hours to test whether another early step of the DENV life cycle is also targeted by PCZ. DENV-2 efficiently bound to the surface of solvent-treated HEK293T cells; however, PCZ treatment 1 hour before and during virus adsorption reduced the levels of DENV-2 bound to the cell surface, as revealed by confocal microscopy and quantified by plaque-forming assay (Figure 3). Therefore, PCZ interferes with DENV-2 binding to the cell surface.

PCZ is a dopamine-receptor antagonist with high affinity for D2R [21]. D2R is widely expressed in various cells and tissues, including neuronal cells, immune cells, and blood vessels [32, 33]. Human and mouse cell lines used in this study also expressed D2R (Supplementary Figure 2) as previously reported [34, 35]. Human and mouse D2R proteins contain 443 and 444 amino acids, respectively, and are highly conserved, with 95% identity at the amino acid level and 89% identity at the nucleotide level. To test whether the inhibitory effect of PCZ on DENV binding is associated with D2R, we first performed an antibody-blockage experiment. Treatment with anti-D2R antibody but not the solvent or control antibody dose-dependently repressed DENV-2 binding (Figure 4A). We further used the D2R-specific ligand N-methylspiperone, commonly used in positron emission tomography to determine D2R distribution [36–39], in a blockage experiment. N-methylspiperone dose-dependently reduced DENV-2 binding (Figure 4B), and its concentration was negatively correlated with levels of virus binding quantified by plaque forming assay (Figure 4C). Thus, DENV-2 binding to the cell surface can be reduced by blockage of D2R with anti-D2R antibody or a D2R-specific ligand.

Next, we established stable D2R-deficient N18 cells by transduction with a lentivirus expressing shRNA-targeting D2R (shD2R). Fluorescently labeled DENV-2 largely colocalized with D2R on the surface of N18 and knockdown control shLacZ-N18 cells (Figure 4D), whereas shD2R-N18 cells lacking D2R expression showed less DENV-2 binding (Figure 4D). Quantification of the cell-bound virus on shD2R-N18 by plaque-forming assay showed approximately 60% reduction as

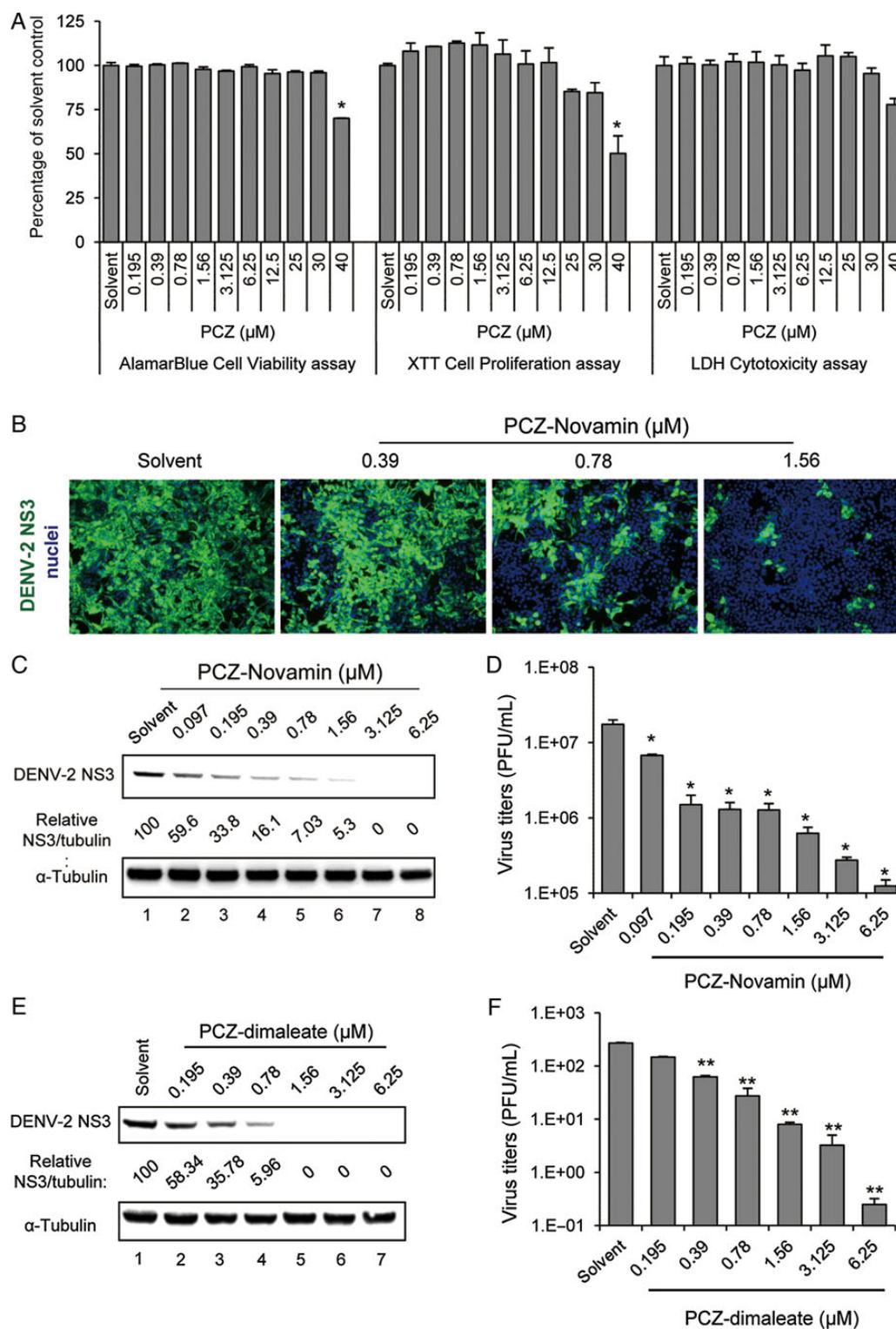


Figure 1. Prochlorperazine (PCZ) exhibits antiviral activity against dengue virus serotype 2 (DENV-2) infection. *A*, PCZ-Novamin cytotoxicity test. HEK293T cells were treated with solvent or the indicated concentrations of PCZ-Novamin for 24 hours. AlamarBlue, XTT, and LDH assays were performed to determine cell viability, cell proliferation, and cytotoxicity, respectively. Data are mean \pm standard deviation of 3 independent experiments. *B*, HEK293T cells were infected with DENV-2 (multiplicity of infection [MOI], 0.1) in the absence (solvent) or presence of PCZ-Novamin for 2 days. Immunofluorescence microscopy (400 \times original magnification) was performed on cells immunostained for DENV-2 NS3 (green) and DAPI for nuclei (blue). HEK293T cells were infected with DENV-2 (MOI, 1) in the absence (solvent) or presence of PCZ-Novamin (*C* and *D*) or PCZ dimaleate (*E* and *F*) for 24 hours. Western blot analysis of protein levels of DENV-2 NS3 and α -tubulin for loading control; relative ratios of NS3 to α -tubulin are adjusted to solvent control (*C* and *E*). Plaque-forming assay of viral progeny production in culture supernatants; data are mean \pm standard deviation of 2 independent experiments (*D* and *F*). * $P < .05$ and ** $P < .01$, compared with solvent control, by the Mann-Whitney U test. Abbreviation: PFU, plaque-forming units.

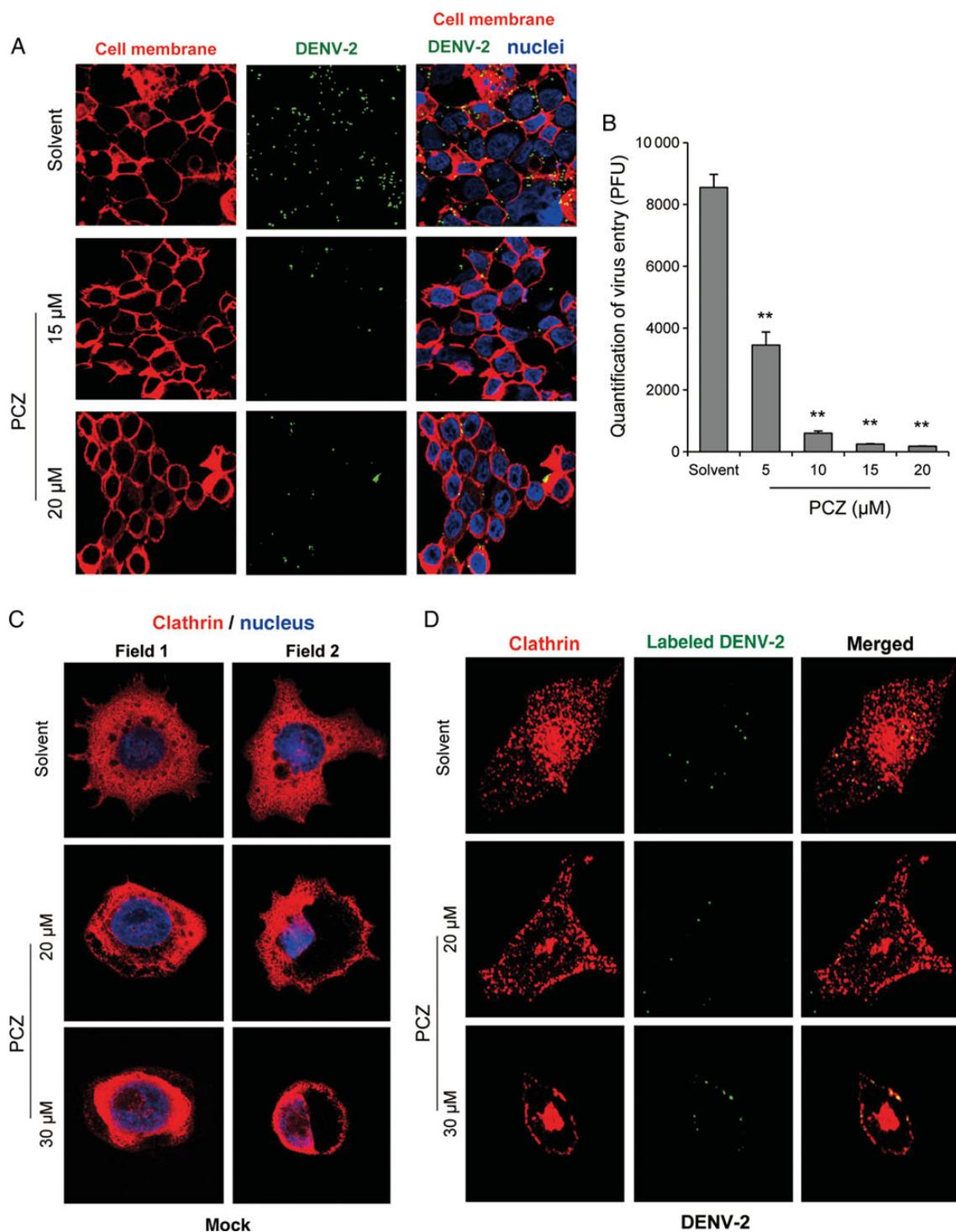


Figure 2. Prochlorperazine (PCZ) inhibits dengue virus serotype 2 (DENV-2) entry into host cells by affecting clathrin distribution. *A*, HEK293T cells were adsorbed with fluorescently labeled DENV-2 (green; multiplicity of infection [MOI], 5) at 4°C for 2 hours. Solvent control or PCZ dimaleate (15 and 20 μM) was added 15 minutes before the end of virus adsorption. After washing with phosphate-buffered saline [PBS], cells were incubated at 37°C for an additional hour, with or without PCZ. Live-cell staining was performed before cell fixation to label cell membrane with CellMask (red) and nuclei with Hoechst (blue). Confocal microscopy images are shown (630× original magnification). *B*, A549 cells adsorbed with DENV-2 (MOI, 5) at 4°C for 2 hours were treated with solvent control or PCZ dimaleate (5 to 20 μM) as described in panel *A*. Cells were physically destroyed to release the internalized DENV-2, and virus was quantified by plaque-forming assay. Data are mean ± standard deviation of 2 independent experiments. ***P* < .01, by the Mann–Whitney *U* test. *C*, Inhibition of DENV-2 entry by PCZ is associated with clathrin distribution. HEK293 cells, mock or adsorbed with fluorescently labeled DENV-2 (green; MOI, 10) at 4°C for 2 hours, were treated with solvent or PCZ dimaleate (20 and 30 μM) for 30 minutes at 37°C. Cells were fixed, permeabilized, and immunostained for clathrin heavy chain (red). Confocal microscopy images of cytoplasmic distribution of clathrin (red) and localization of labeled DENV-2 (green) are shown (630× original magnification). Abbreviation: PFU, plaque-forming units.

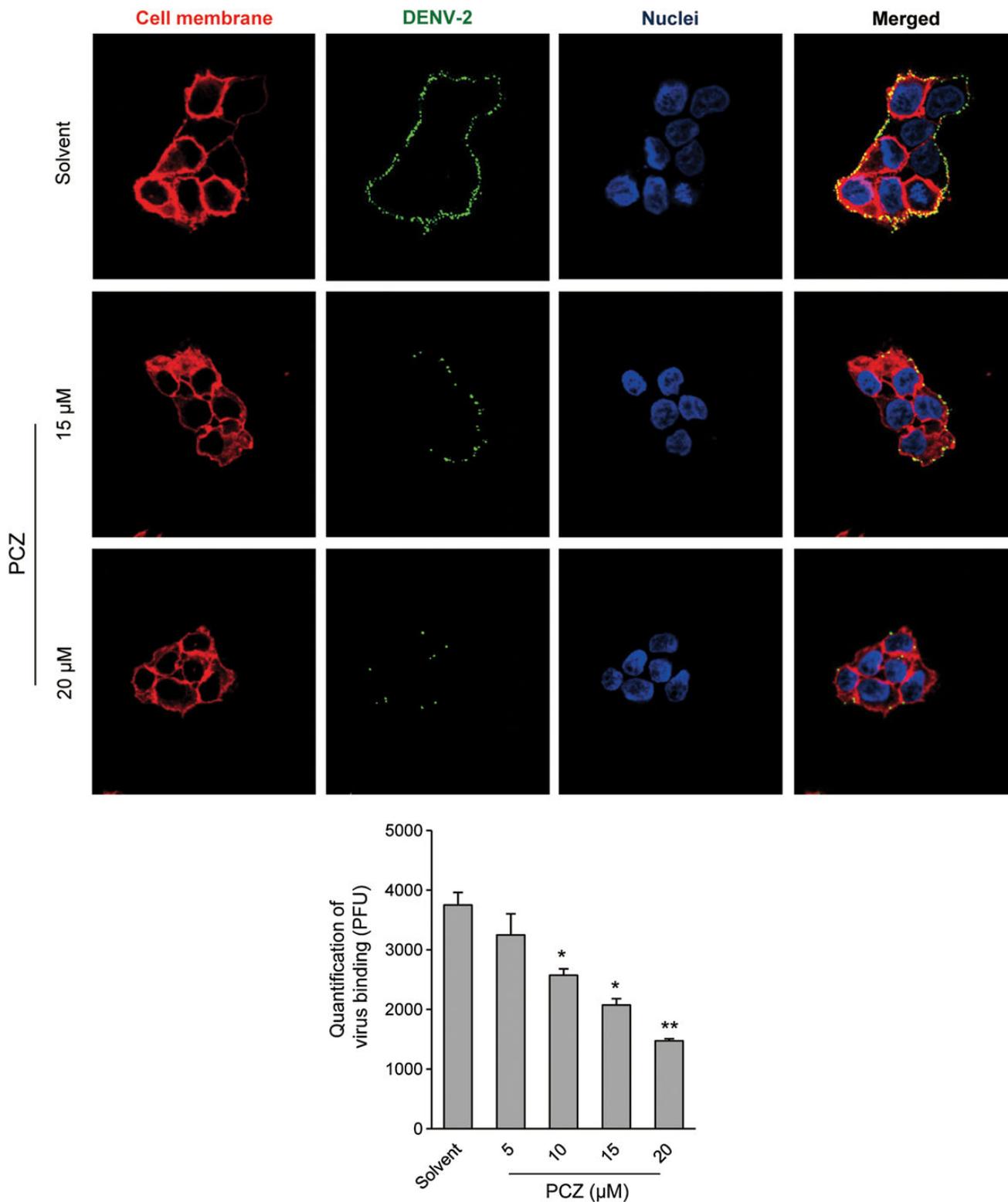


Figure 3. Prochlorperazine (PCZ) interferes with dengue virus serotype 2 (DENV-2) binding onto the cell surface. HEK293T cells pretreated with the indicated doses of PCZ dimaleate for 1 hour were subsequently adsorbed with fluorescently labeled DENV-2 (green; multiplicity of infection [MOI], 5) at 4°C for 2 hours in the absence (solvent) or presence of PCZ. Cells were then stained with CellMask for cell membrane (red) and Hoechst for nuclei (blue). Confocal microscopy images are shown (630× original magnification). For quantification, A549 cells pretreated with PCZ dimaleate for 1 hour were adsorbed with DENV-2 (MOI, 5) at 4°C for 2 hours and physically destroyed to release the adsorbed DENV-2. The amount of cell-bound virus was determined by plaque-forming assay. Data are mean ± standard deviation of 2 independent experiments. * $P < .05$ and ** $P < .01$, by the Mann–Whitney U test.

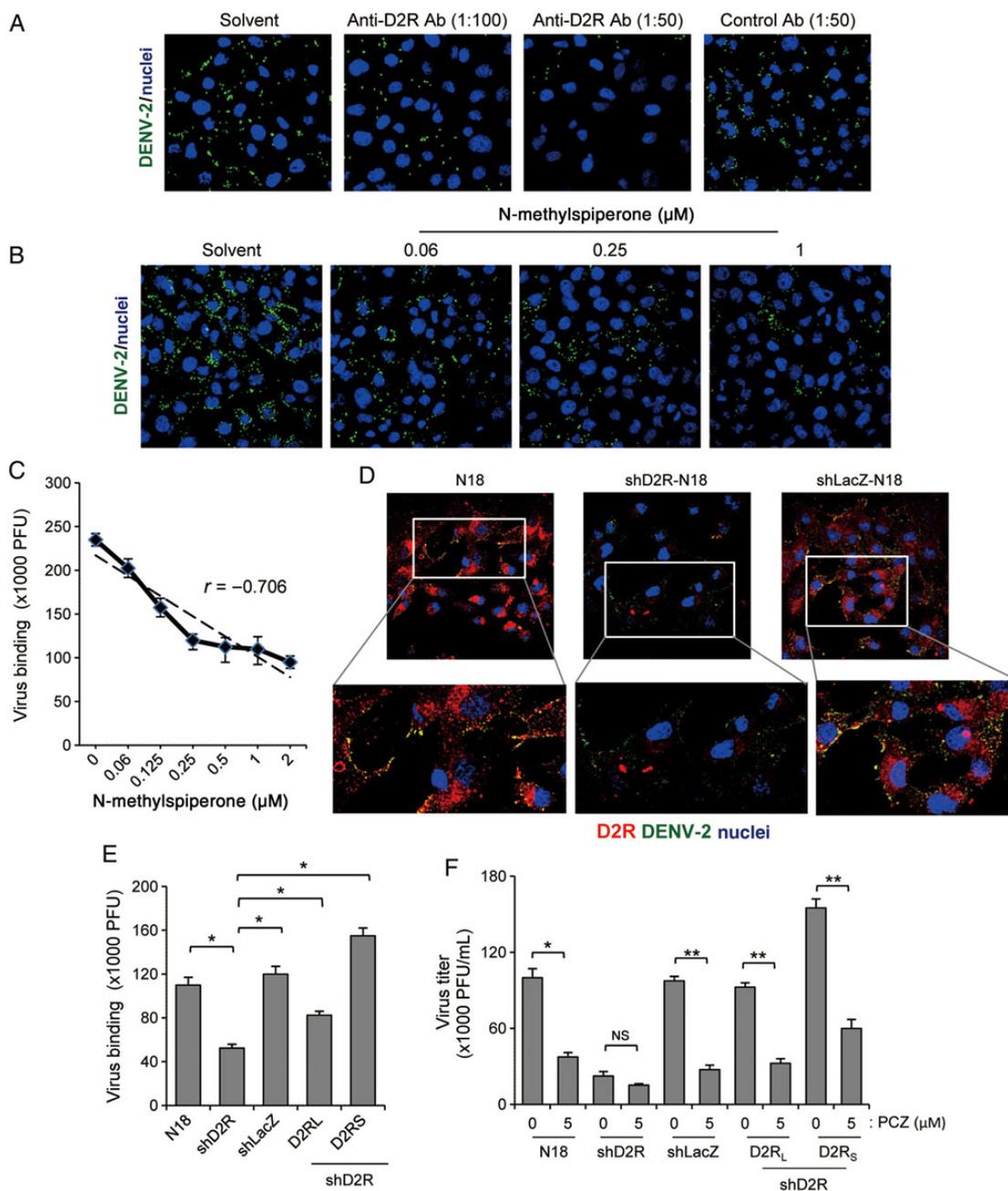


Figure 4. Dengue virus serotype 2 (DENV-2) binding to cell surface is associated with dopamine D2 receptor (D2R). *A*, Antibody (Ab) against D2R decreased DENV-2 binding onto cell surfaces. Fluorescently labeled DENV-2 (green; multiplicity of infection [MOI], 5) was bound to N18 cells at 4°C for 2 hours with or without anti-D2R Ab (1:100 and 1:50 dilutions). Rabbit immunoglobulin G Ab (1:50) was an Ab control. Confocal microscopy images are shown (400× original magnification). *B*, D2R-specific ligand N-methylspiperone reduced DENV-2 binding onto cell surfaces. Fluorescently labeled DENV-2 (green; MOI, 40) was bound to N18 cells at 4°C for 30 minutes with or without the indicated doses of N-methylspiperone. Confocal microscopy images are shown (400× original magnification). *C*, Level of virus binding is negatively correlated with the dose of N-methylspiperone. DENV-2 (MOI, 50) was bound to N18 cells at 4°C for 30 minutes with or without N-methylspiperone. Cells were physically destroyed to release the adsorbed DENV-2. The amount of cell-bound virus was determined by plaque-forming assay. Data are mean ± standard deviation of 2 independent experiments. The Pearson correlation coefficient (r) is presented. *D–F*, Knock-down of D2R expression reduces DENV-2 binding. *D*, Cells were adsorbed with fluorescently labeled DENV-2 (green; MOI, 10) at 4°C for 2 hours. Without permeabilization, cells were immunostained for D2R (red) and Hoechst for nuclei (blue). Confocal microscopy images are shown (630× original magnification). *E*, DENV-2 binding (MOI, 5) was performed on the indicated D2R-deficient and -rescued N18 cells at 4°C for 2 hours. Cells were then physically destroyed to release the adsorbed virus to be quantified by plaque-forming assays. *F*, The inhibition of DENV-2 binding by prochlorperazine (PCZ) depends on D2R. Cells were pretreated with solvent or 5 μM PCZ dimaleate for 30 minutes and adsorbed with DENV-2 (MOI, 5) at 4°C for 2 hours. After washing with cold serum-free medium, cells were shifted to 37°C for overnight incubation in the absence of PCZ. Plaque-forming assay of progeny production in culture supernatant; data are mean ± standard deviation of 2 independent experiments. * $P < .05$ and ** $P < .01$, by the Mann–Whitney U test. Abbreviations: NS, not significant; PFU, plaque-forming units.

compared to N18 and shLacZ-N18 controls (Figure 4E). There are 2 isoforms of D2R: the long form (D2R_L) and the short form (D2R_S), which are generated by alternative splicing and differ by 29 amino acids in the third intracellular loop [35]. We rescued the D2R protein expression of shD2R-N18 cells by overexpressing a shD2R-resistant long or short isoform of D2R generated by introducing wobble mutations. The protein expression of D2R was increased in the rescued cells to a level similar to that of controls (Supplementary Figure 2B). Notably, the amount of virus binding was significantly higher in the D2R-rescued cells as compared with the D2R-knockdown cells (Figure 4E). Finally, we tested whether the antiviral effect of PCZ on DENV binding depended on D2R. Cells were pretreated with PCZ for 30 minutes, then DENV-2 was adsorbed at 4°C for 2 hours. After extensive washing, cells were incubated overnight at 37°C without PCZ. PCZ had an antiviral effect in cells with D2R expression (N18, shLacZ-N18, shD2R-D2R_L-N18, and shD2R-D2R_S-N18) but not D2R-knockdown shD2R-N18 cells (Figure 4F). Thus, the inhibitory effect of PCZ on DENV binding was associated with D2R.

PCZ Protects DENV-2–Induced Lethality in Animal Models

We then used a mouse challenge model to test whether PCZ has an in vivo antiviral property. STAT1 is a transcription factor activated by many cytokines, and disruption of the mouse *Stat1* gene results in compromised innate immunity against viral diseases [40]. We previously developed a DENV model in *Stat1*^{-/-} mice that showed paralysis, hemorrhage, vascular leakage, and death after challenge with a mouse-adapted DENV-2 NGC-N strain [30, 31]. PCZ is available for the ingestion route (tablet or capsule) and parenteral route (injection). We tested both routes, with PCZ dimaleate and PCZ-Novamin, respectively, in our animal model. *Stat1*^{-/-} mice challenged with 5 × 10⁴ PFU/mouse of DENV-2 received PCZ dimaleate solution (1 and 5 mg PCZ/kg body weight/day) orally for 10 days, except the first treatment was given by intraperitoneal injection, starting either immediately or at 6 hours after DENV-2 infection for the prophylactic and therapeutic modes of treatment, respectively. A high dose of immediate PCZ treatment (5 mg/kg body weight/day) completely protected against death due to DENV-2 infection, while 90% of the vehicle control mice died (Figure 5A). Immediate treatment with a lower dose (1 mg PCZ/kg body weight/day) delayed animal mortality (T₅₀, 36 days vs 18 days for the vehicle control), with a moderate effect on overall animal survival. In the therapeutic model (6-hour-delay group), PCZ treatment (5 mg PCZ/kg body weight/day), starting 6 hours after infection and ending 10 days later, delayed death and improved the overall survival to 50% of DENV-2–inoculated animals.

We further evaluated the anti-DENV efficacy of PCZ-Novamin administered by intraperitoneal injection with a 2-day interval. All mice in the vehicle control group died within 10 days of

DENV-2 challenge (Figure 5B), earlier than the control mice (Figure 5A). The route and frequency of administration, oral feeding twice per day, compared with intraperitoneal injection every 2 days, may have resulted in the different T₅₀ in the control mice displayed in Figure 5A and 5B (18 vs 6 days). Oral intervention with PBS in Figure 5A may protect mice against dehydration, allow them to develop some immune responses, and result in longer survival. Immediate treatment with 8 or 4 mg PCZ/kg body weight every 2 days protected mice against DENV-2 challenge during the 10-day treatment period. Even after the treatment stopped at day 10 after infection, mice receiving 8 mg PCZ remained healthy, whereas some of the mice receiving 4 mg PCZ died (40% survival). An even lower dose, 2 mg PCZ/kg body weight every 2 days, slightly delayed the DENV-2–induced lethality, with an overall survival of 20%. In the therapeutic mode of treatment, starting from 6 hours after infection and ending at day 10 after infection, 40% of mice receiving 8 mg PCZ/kg body weight every 2 days survived the DENV-2 challenge, but a lower dose (4 mg/kg body weight every 2 days) of PCZ did not improve the overall survival. Thus, PCZ has a dose-dependent protective effect on DENV-2 challenge in delaying the lethality and improving overall survival. We further measured the viremia levels in sera collected from mice with or without PCZ treatment. Viremia was detected in PBS control mice after 2 days of DENV-2 infection (Figure 5C), but the 6-hour delay in initiation of treatment (8 mg PCZ-Novamin/kg body weight) significantly reduced the viremia levels. Furthermore, immediate treatment with 8 mg of PCZ-Novamin reduced viral loads below the detectable level. Thus, consistent with the mouse survival results, viral load data demonstrated the antiviral activity of PCZ against DENV infection.

DISCUSSION

We selected PCZ to test its potential as an accelerated anti-DENV drug candidate after taking into account that (1) PCZ showed anti-HCV activity in cell-based screening [22]; (2) HCV and DENV, members of the *Flaviviridae* family, share similarities in life cycles and host–pathogen interaction profiles [23, 24, 26–28]; (3) PCZ may relieve symptoms associated with DENV infection, such as headache, nausea, and vomiting; and (4) PCZ is a clinically approved drug available for human use. Indeed, PCZ exhibited in vitro and in vivo antiviral activity against DENV-2 infection.

Regarding the mode of action, PCZ likely targets 2 steps of the DENV life cycle, binding and entry, through different mechanisms. Because chlorpromazine, a compound with structure similar to that of PCZ, is known to affect clathrin distribution [25, 33], it was not surprising to observe that PCZ could block DENV entry through clathrin-mediated endocytosis (Figure 2). Interestingly, DENV binding was affected by

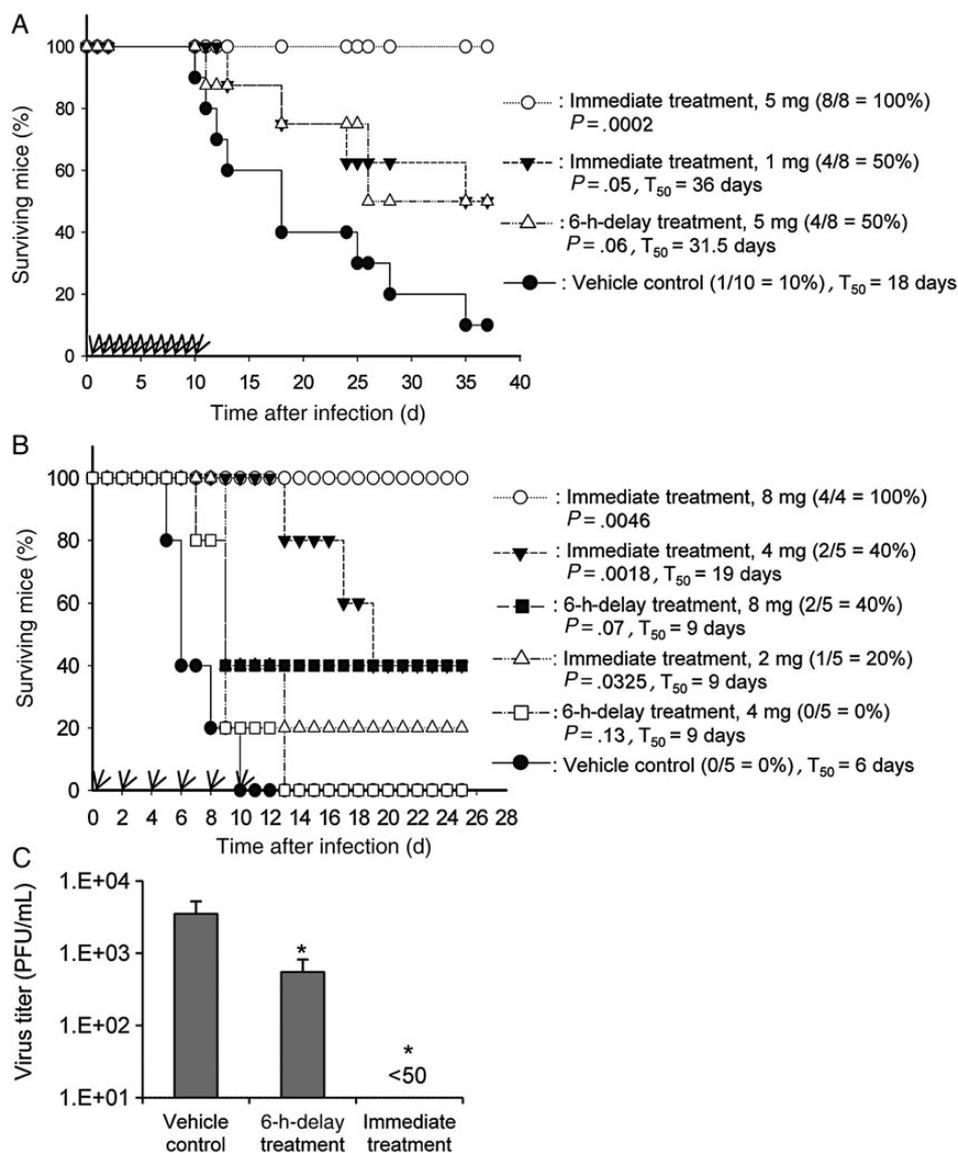


Figure 5. Prochlorperazine (PCZ) protects against dengue virus serotype 2 (DENV-2)-induced lethality in an animal model. The protective effect of PCZ dimaleate (A) and PCZ-Novamin (B and C) was tested with immediate and 6-hour-delay treatments. A, *Stat1*^{-/-} mice received phosphate-buffered saline (PBS; vehicle control; n = 10) or either 5 mg (n = 8) or 1 mg (n = 8) of PCZ dimaleate/kg body weight intraperitoneally at the time of infection (immediate treatment) or 5 mg of PCZ dimaleate/kg body weight intraperitoneally at 6 hours after infection (6-hour-delay treatment; n = 8). Mice received follow-up treatments with PBS or half the dose of PCZ dimaleate orally every 12 hours until day 10 after infection. B, *Stat1*^{-/-} mice received PBS (n = 5) or either 8 mg (n = 4), 4 mg (n = 5), or 2 mg (n = 5) of PCZ-Novamin/kg body weight intraperitoneally at the time of infection (immediate treatment) or 8 mg (n = 5) or 4 mg (n = 5) of PCZ-Novamin/kg body weight intraperitoneally at 6 hours after infection (6-hour-delay treatment). Mice then received follow-up treatments at 2-day intervals with the same dose of PCZ-Novamin intraperitoneally until day 10 after infection. The days with treatment are marked by arrows. Mice survival was presented as percentage of survival. The median survival time (T_{50}) and P value, by the log-rank test, are presented. C, *Stat1*^{-/-} mice received PBS (vehicle control; n = 4) or 8 mg of PCZ-Novamin/kg body weight intraperitoneally at the time of infection (immediate treatment; n = 4) or at 6 hours after infection (6-hour-delay treatment; n = 4). Serum samples were collected at day 2 after infection, and virus loads were determined by plaque-forming assay. Data are mean \pm standard deviation. * $P < .05$, by the Mann-Whitney U test. Abbreviation: PFU, plaque-forming units.

PCZ in a D2R-dependent manner (Figure 3), because DENV binding to the cell surface could be blocked by anti-D2R antibody, D2R-specific ligand, and knockdown of D2R expression (Figure 4). Other D2R antagonists could also repress DENV-2 infection (Supplementary Figure 3A and 3B), although their

efficacy varied, probably because of their drug affinity to D2R and their effect on clathrin distribution [21, 25, 33, 41]. However, SCH23390, a highly potent and selective D1R antagonist [33], had minor antiviral effects against DENV-2 infection in HEK293T and N18 cells at relatively high doses

(Supplementary Figure 3C and 3D); thus, D2R rather than D1R is likely to participate in DENV infection. This notion was supported by a recent study showing an interplay between DENV-2 and another subtype of D2-like receptor, dopamine D4 receptor (D4R), in the early viral life cycle [42]. Collectively, PCZ can block DENV-2 binding to D2R on the cell surface and also viral entry by inhibiting clathrin-mediated endocytosis. Because PCZ targets 2 distinct cellular components but not the viral proteins, drug-resistant virus strains are not likely to develop with use of PCZ, especially with short-term treatment, as was reported for a D4R antagonist [42].

The US Food and Drug Administration lists PCZ as an active pharmaceutical ingredient approved on the basis of an abbreviated new drug application and conveys its safety information and pharmacodynamics in both humans and animals. In the material safety data sheets for PCZ [43, 44], the LD₅₀ in mice has been described as 191 and 400 mg/kg body weight for intraperitoneal injection and oral administration, respectively. Thus, the doses of PCZ showing in vivo protective effect against DENV-2 infection in our animal model are well below the potentially hazardous doses. The equivalent dose of 5 mg PCZ/kg/day for mice is 0.405 mg/kg/day for humans, as calculated by a published method [45]; thus, for a 60-kg person, the dose would be 24.3 mg/day. The clinical dose of PCZ maleate recommended to prevent nausea and vomiting is 5–10 mg/dose 2–3 times daily and, for treating nausea and vomiting, is 20 mg followed by 10 mg 2 hours later if required [46]. Therefore, reaching the noted protective dose of PCZ in humans is feasible. However, detailed pharmacokinetic study and antiviral tests in humans are required to find the best regimen for PCZ in dengue-related diseases.

So far, a specific antiviral agent blocking DENV infection is lacking, and supportive care and symptomatic treatments are commonly used to treat dengue. Our finding that PCZ, a drug widely used to relieve anxiety, headache, nausea, and vomiting, common symptoms among patients with dengue [41, 47–49], displays potent antiviral activity against DENV infection is novel and significant. PCZ treatment in patients with dengue might have 2 beneficial effects: blocking DENV infection and relieving clinical symptoms. Because dengue is an acute disease, the window and length of therapeutic treatment is narrow and short. A compound in clinical use that has anti-DENV activity, such as PCZ, might then be prophylactically prescribed to high-risk individuals and/or patients with fever before dengue diagnosis in areas of epidemicity during dengue outbreaks. Another potential advantage of PCZ treatment in patients with dengue fever is to prevent the development of dengue hemorrhagic fever and dengue shock syndrome, because a higher magnitude of DENV replication is associated with severe dengue disease. Overall, we reveal that PCZ has a previously unknown antiviral function and could be further clinically evaluated as a potential therapeutic and prophylactic agent against DENV infection.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Y. S. and Y.-L. Lin designed research, analyzed data, and wrote the manuscript. Y. S., J.-J. L., and Y.-L. Lee performed research and analyzed data.

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Potential conflicts of interest. All authors: No reported conflicts.

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