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Discovery and Optimization of 2,4-Diaminoquinazoline Derivatives as a New Class of Potent Dengue Virus Inhibitors

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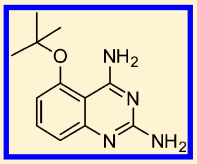
Supporting Information

ABSTRACT: The results of a high-throughput screening assay using the DENV-2 replicon showed that the 2,4-diaminoquinazoline derivative **4a** has a high dengue virus inhibitory activity (EC_{50} = 0.15 μM). A series of 2,4-diaminoquinazoline derivatives based on **4a** as a lead compound were synthesized and subjected to structure−antidengue activity relationship studies. Among the series of 2,4-diaminoquinazoline derivative probed, **4o** was observed to display both the highest antiviral potency (EC_{50} = 2.8 nM, SI > 1000) and an excellent pharmacokinetic profile.



INTRODUCTION

Dengue virus (DENV) belongs to the *Flavivirus* genus of the *Flaviviridae* family,[1] whose genome is comprised of a 10.7 kb, single, positive-stranded RNA. Four serotypes of this virus, known as DENV-1−DENV-4,[2] exist. DENV, primarily transmitted by *Aedes* mosquitoes, is the cause of dengue fever, an infectious tropical disease that the World Health Organization[3] has reported to afflict 40−80 million people every year. With increased levels of urbanization, population growth, migration, and international travel, as well as difficulties associated with effective vector control,[4] incidences of the DENV illness have increased 30-fold in the last 50 years.

The current management of dengue infections focuses on the treatment of symptoms, which often can be a tedious and intensive process.[5] Because neither drugs nor vaccines are available to combat dengue viral infections, explorations to uncover small molecules that have potent antidengue bioactivities are highly important. In recent years,[5−32] a number of antiviral agents with this property have been discovered, including the *N*-sulfonylanthranilic acid derivate **1**,[18] adenosine analogue **2**,[9] and chlorophenyl-thiophene derivate **3**.[19] However, none of these substances have entered into clinical trials.

In contrast to protein-based in vitro screening approaches, those that employ replicon cell-based screening are useful in both the discovery of novel antiviral targets and the confirmation of the activities of agents in infectious virus systems. In previous studies, we applied a dengue replicon cell line in combination with a robotic high-throughput system to screen an available compound library. In this effort, an orthogonal cocktail library[33] was explored for dengue replication inhibition activity using a dengue replicon cell line-based assay developed by Garcia.[34] By using this approach, we identified 5-methoxyquinazoline-2,4-diamine (**4a**, Figure 1) as a lead compound that

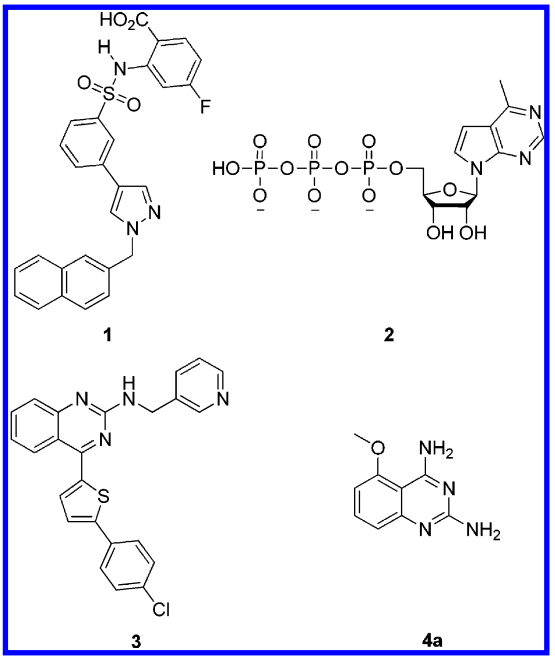


Figure 1. Recently uncovered DENV inhibitors and **4a**.

serves as a modest inhibitor of DENV-2 replicon with a half maximal effective concentration (EC_{50}) value of 0.15 μM and a low cytotoxicity [50% cytotoxicity concentration (CC_{50}) > 10 μM]. Substances containing the core structure of **4a** are known to possess a broad spectrum of biological activities, such as protein lysine methyltransferase G9a inhibition,[35−37] *SMN2* promoter

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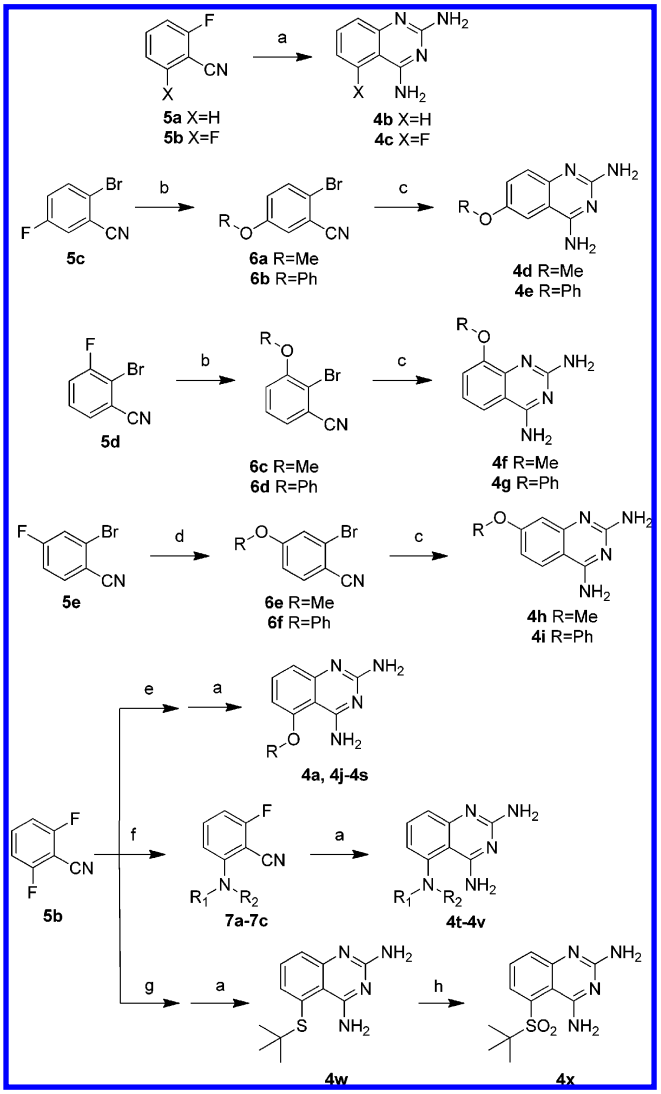
activation,[38,39] dihydrofolate reductase inhibition,[40−49] and others.[50−52] Utilizing **4a** as the lead compound, we initiated a study to explore structure−dengue viral activity relationships of 2,4-diaminoquinazoline derivatives. This effort led to the identification of **4o** as a highly potent (EC_{50} = 2.8 nM) and relatively nontoxic (CC_{50} > 10 μM) dengue antiviral agent. As far as we are aware, this substance is the most potent inhibitor uncovered to date using a cell-based DENV replicon assay.[5,6,8−13,15−17,19,20,26,31,32]

CHEMISTRY

The general procedure employed for the synthesis of members of the 2,4-diaminoquinazoline derivative series is shown in Scheme 1. On the basis of this approach, we prepared a range of substances in this family that contain variously substituted phenyl rings to examine structure−activity relationships (SARs). For this purpose, 2,4-diaminoquinazoline (**4b**) and 5-fluoro-2,4-diaminoquinazoline (**4c**) were synthesized by utilizing cyclization reactions of 2-fluorobenzonitrile and 2,6-difluorobenzonitrile, respectively, with guanidine carbonate. To replace substituents at different positions selectively, the respective 3-, 4-, and 5-fluoro benzonitrile derivatives **5c−5e** were reacted with sodium methoxide or phenol to produce the corresponding aryl ethers. The benzonitriles were then subjected to Ullmann reactions using guanidine carbonate to provide the methoxy- or phenoxy-substituted 2,4-diaminoquinazolines **4d−4i**. Reaction of 2,6-difluorobenzonitrile (**5b**) with sodium methoxide or phenol followed by Ullman cyclization with guanidine carbonate gave the 5-methoxy- and -phenoxy-substituted 2,4-diaminoquinazolines **4a** and **4j**. In a similar manner, the 5-alkoxy 2,4-diaminoquinazolines **4k−4s** were produced from 2,6-difluorobenzonitrile **5b** utilizing reactions with alkoxide anions derived from the corresponding alcohols followed by condensation with guanidine carbonate. The 5-amido-2,4-diaminoquinazolines **4t−4v** were prepared using initial reactions of **5b** with the corresponding amines under microwave irradiation conditions followed by condensation reactions with guanidine. Finally, reaction of **5b** with *tert*-butylthiol followed by condensation with guanidine gave **4w**, which upon oxidation with oxone afforded **4x**.

Scheme 1[a]

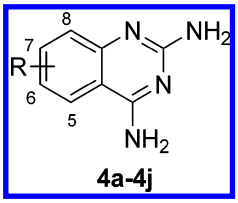


[a]Reagents and conditions: (a) Guanidine carbonate, DMA, 140 °C, 8 h. (b) For **6a** and **6c**: NaOMe, DMF, room temperature, 5 h; for **6b** and **6d**: phenol, K_2CO_3, DMF, 100 °C, 8 h. (c) Guanidine carbonate, CuI, K_2CO_3, NMP, 155 °C, 5 h. (d) For **6e**: NaOMe, THF, 50 °C, 6 h; for **6f**: phenol, K_2CO_3, DMF, 45 °C, 6 h. (e) For **4a**: NaOMe, THF, room temperature, 6 h; for **4j**: phenol, K_2CO_3, DMF, 45 °C, 6 h; for **4k−4s**: alcohols, NaH, THF, room temperature, 6 h. (f) Amines, K_2CO_3, DMSO, microwave (110 °C, 15 min). (g) *tert*-Butylthiol, K_2CO_3, DMF, room temperature, 10 h. (h) Oxone, MeOH/H_2O, room temperature, 12 h.

RESULTS AND DISCUSSION

The results of experiments probing the BHK-DENV-2-replicon inhibitory effects of **4a−4j** are displayed in Table 1. The findings show that **4b** and **4c**, in which the methoxy group in **4a** is removed from phenyl ring or replaced by a C-5 fluorine, are devoid of DENV inhibitory activity. Moving the methoxy to other positions of the phenyl ring provided **4d**, **4f**, and **4h**. In addition, relocation of the methoxy substituent to give **4d**, **4f**, and **4h** results in a significant reduction inhibitory activity. In contrast to **4a**, **4j** with a C-5 phenoxy substituent exhibits a 5-fold higher bioactivity. Furthermore, analogues **4e** and **4i**, containing phenoxy substituents at positions 6 and 7, do not

Table 1. BHK-DENV-2-Replicon Inhibitory Effects of Substituted 2,4-Diaminoquinazolines 4a−4j



compd	R	CC_{50} (μM)	BHK-DENV-2-replicon EC_{50} (μM)	SI
4a	5-OMe	>10	0.15	>66.7
4b	H	>10	>10	
4c	5-F	>10	>10	
4d	6-OMe	>10	>10	
4e	6-OPh	>10	>10	
4f	8-OMe	>10	5.4	>1.9
4g	8-OPh	0.55	0.060	9.2
4h	7-OMe	>10	>10	
4i	7-OPh	>10	>10	
4j	5-OPh	>10	0.029	>300

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serve as inhibitors of DENV. Although the 6-methoxy- and 6-phenoxy-substituted derivatives **4f** or **4g** are inhibitors, their activities are lower than those of the similarly substituted analogues **4a** or **4j**. In addition, **4g** was observed to exhibit cytotoxicity (CC_{50} = 0.55 μM). The overall findings indicate that electron-donating substituents at position 5 of the aryl ring of 2,4-diaminoquinazolines are crucial for BHK-DENV-2-replicon inhibitory activity and that the steric character of the substituents influences activity.

The results summarized above led to a study that targeted further optimization of inhibitory activity by varying substituents at position 5. We observed (Table 2) that a moderate variation of the alkyl chain in the alkoxyl group of **4a** (as in **4k** and **4l**) leads to improved bioactivity. However, introduction of the longer octyl group at this position results in a decreased inhibitory activity and increased cytotoxicity (e.g., **4m** vs **4a** or **4k**/**l**). Additionally, when the ethoxy group in **4k** is replaced by a 2,2,2-trifluoroethoxy group (e.g., **4n**), the value EC_{50} is improved to 7.9 nM.

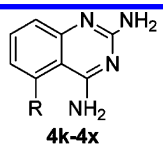
This finding suggested that the presence of a more sterically bulky rather than longer alkyl group at position 5 would lead to improved bioactivity. As anticipated, the 2,4-diaminoquinazoline derivative **4o**, possessing a 5-*tert*-butoxy group, was found to be the most potent inhibitor (EC_{50} = 2.8 nM) in this series. However, a change of the *tert*-butoxy to a neopentyloxy group (e.g., **4p**) causes a significant decrease in activity and increase in toxicity. This observation demonstrates the crucial role played by the steric bulk and lipophilicity of the C-5 substituent. Another example of these effects is found in the 5-cyclopentyloxy derivative **4q**, which was observed to have an EC_{50} value of 7.4 nM, that is more potent than that of the cyclohexyloxy analogue **4r** (EC_{50} = 0.13 μM). The combined results suggest the viral target has a spatially restricted conformation that accommodates a narrow range of C-5 substituents.

The 5-benzyloxy derivative **4s** was found to have a antiviral activity that is similar to the corresponding phenoxy derivative **4j**. In contrast, introduction of amine groups at C-5 of the 2,4-diaminoquinazoline structure has a deleterious effect on viral inhibitory activity. For example, in comparison to **4o**, the *tert*-butylamino analogue **4t** has a low activity and increased toxicity (CC_{50} = 2.5 μM). In addition, introduction of a secondary or cyclic amine group (e.g., **4u** and **4v**), as well as a *tert*-butylthio group (**4w**), causes a significant decrease in inhibitory activity. Finally, the presence of the electron-withdrawing *tert*-butylsulfonyl group at C-5 (**4x**) results in a complete loss of activity. These observations indicate that electronic and steric effects of the C-5 substituent in the 2,4-diaminoquinazolines play important roles in governing antiviral activity and cytotoxicity. Most significantly, the effort led to the identification of **4o** as a highly promising and potent BHK-DENV-2-replicon inhibitor.

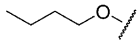
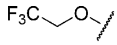
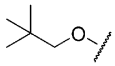
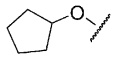
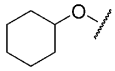
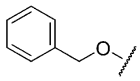
The results of comparisons of **4o** with the two reference compounds Riba (ribavirin) and MPA (mycophenolic acid) using the functional replicon screening system are shown in Figure 2A. Diaminoquinazoline **4o** was found to be an effective inhibitor of the reporter signal at a concentration that is much lower than those of the two reference compounds. The results of studies, in which **4o** was screened using an infectious DENV-2 virus (Experimental Section: Infectious DENV-2 Virus Test), confirm its high antiviral activity. The observations show that **4o** efficiently inhibits multiplication of the virus in BHK cells and intracellular viral expression. In addition, this substance lowers the percentage of viral NS1 antigen positive cells, and as a result, it prevents the spread of virus in host cell cultures (EC_{50} < 0.05 μM) (Figure 2B). Finally, **4o** also exhibits strong antiviral activity in the highly susceptible C6/36 cell line, showing that it inhibits the formation of the virus-induced cytopathic effect (arrows) that is indicative of active viral multiplication (Figure 3).

The antiviral potency of **4o** is validated by observation of its activity in two different cell lines, including the DENV-1 replicon as well as the DENV-2 wild-type virus. However, these findings do not enable identification of the mode of action of **4o**. Potential targets of this substance could be either viral proteins or common cellular components required for virus multiplication. Because **4o** exerts antiviral activity in both the replicon and the infectious systems, it might be acting by inhibiting viral intracellular replication cycles, which could effect steps occurring between viral RNA replication, viral protein translation, and protein cleavage and post-translational modification, or alternatively, it could target one of a number of cellular signal pathways. While recognizing the structural similarities that exist between **4o** and known DHFR inhibitors, its actual mode of action remains unknown.[40−49] The observa-

Table 2. BHK-DENV-2-Replicon Inhibitory Effects of Substituted 2,4-Diaminoquinazolines 4k−4x



4k-4x

Compound	R	CC_{50}(μM)	BHK-DENV-2-Replicon EC_{50}(μM)	SI
4k		>10	0.037	>200
4l		>10	0.036	>200
4m		0.73	0.18	4.1
4n		>10	0.0079	>1000
4o		>10	0.0028	>1000
4p		0.65	0.080	8.1
4q		>10	0.0074	>1000
4r		>10	0.13	>76.9
4s		>10	0.039	>200
4t		2.5	0.0092	>200
4u		>10	3.3	>3.0
4v		1.2	0.15	8
4w		5.3	0.90	5.9
4x		>10	>10	/

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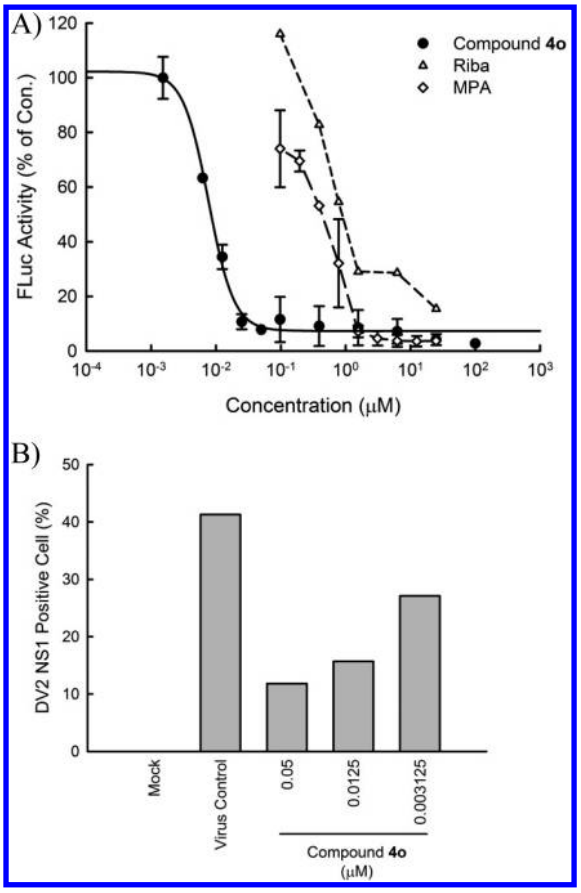

Figure 2. (A) Antiviral activity of **4o** and reference compounds in BHK-DV2-replicon cell line. (B) Validation of **4o** antiviral activity using an infectious DENV-2 virus in BHK cells.

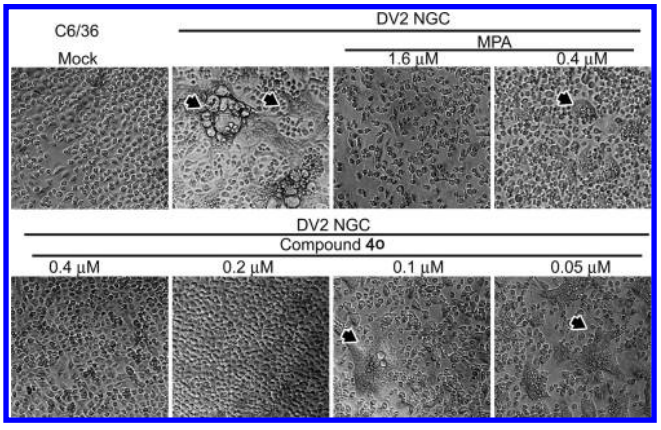

Figure 3. Antiviral activity of **4o** in C6/36 cell line infected by DENV-2 virus.

tion that the cytotoxic effect of **4o** is less than 20% in comparison with its antiviral activity of 80% at the same concentration suggests that its action to deplete cellular ATP is likely a consequence of the reduction of the cell proliferation rate and not of cytotoxicity, which would have been detected using the MTT method. Therefore, **4o** possesses a unique anti-DV activity in addition to its possible DHFR inhibitory effect.

The pharmacokinetic profile of **4o** was evaluated in rats (Table 3). The results show that this substance exhibits a high oral bioavailability (69%) and plasma clearance (6.56 mL/min/kg). Following an oral dose of 20 mg/kg, the rat plasma concentration of **4o** reaches a maximum (249 ng/mL) at 2.0 h postdose administration. The high oral bioavailability (69%) and long half-life ($t_{1/2}$ = 5 h) indicate that **4o** has high potential for development as an orally active, antidengue virus candidate. In addition, **4o** has an IC_{50} of 20.2 μM for blockade of the I_{kr} potassium channel hERG (human ether-a-go-go-related gene) on HEK-293 cells determined by using a patch clamp assay.

CONCLUSION

The results of a structure−activity investigation of 2,4-diaminoquinazoline derivatives as potent antidengue virus agents is described above. The SAR led to the discovery of **4o**, which is the most potent (EC_{50} = 2.8 nM) and selective (SI > 1000) DENV inhibitor uncovered to date. The results of the effort showed that the bulky and electron-donating C-5 *tert*-butoxy group of **4o** is crucial for its excellent inhibitory activity. Confirmation of its antiviral activity and a determination of its pharmacokinetic profile show that **4o** is as an attractive candidate for further studies being carried out in our laboratory aimed at elucidating its novel mechanism and in vivo characteristics.

EXPERIMENTAL SECTION

Chemistry. ^{1}H nuclear magnetic resonance (NMR) spectral data were recorded using $CDCl_3$, DMSO-d_6, or MeOH-d_4 solutions with a Varian Mercury-VX 400 or 300 NMR spectrometer, and ^{13}C NMR spectra were recorded using DMSO-d_6 solutions on a Bruker DRX-400 NMR spectrometer. Chemical shifts (δ) are reported in parts per million (ppm), and the signals are described as brs (broad singlet), s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), and m (multiplet). Coupling constants (J values) are given in Hz. Low-resolution (MS) and high-resolution (HRMS) mass spectra were recorded at an ionizing voltage of 70 eV on a Waters Micromass GCT Premier spectrometer. Column chromatography was conducted using silica gel (200−300 mesh). All reactions were monitored using thin-layer chromatography (TLC) on silica gel plates. The purity (>95%) of each substance prepared in this study was determined using chromatographic analysis with an Agilent 1200 series LC system (Agilent ChemStation Rev.B.03.01); column, ZORBAX Eclipse XDB-C18, 4.6 mm × 150 mm, 5 μm, or Nova Pak C18 3.9 mm × 150 mm, 4 μm; mobile phase, acetonitrile (MeCN)/H_2O (0.2% triethylamine); flow rate, 1.0 mL/min; UV wavelength, maximal absorbance at 254 nm; temperature, ambient; and injection volume, 5 μL (see Table S1 in the Supporting Information). All microwave irradiation reactions were conducted by using CEM E-48 microwave reactor.

*Quinazoline-2,4-diamine (**4b**).* A mixture of 2-fluorobenzonitrile (**5a**, 100 mg, 0.83 mmol) and guanidine carbonate (149 mg, 0.83 mmol) in 3 mL of *N*,*N*-dimethylacetamide (DMA) was stirred at 140 °C for 8 h, then cooled to room temperature, and diluted with water (40 mL). The mixture was extracted with dichloromethane (DCM) (3 × 40 mL). The extracts were dried over Na_2SO_4 and concentrated in vacuo to give a residue that was subjected to silica gel chromatography (8:1 DCM/MeOH, 1% NH_3·H_2O) to afford **4b** as a colorless powder (102 mg, 77%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 6.55 (brs, 2 H), 7.05−7.16 (m, 1 H), 7.25 (d, J = 8.50 Hz, 1 H), 7.50−7.60 (m, 1 H), 7.75 (brs, 2 H), 8.02 (d, J = 8.50 Hz, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 110.37, 119.80, 123.56, 124.23, 132.37, 152.51, 160.78, 162.50. HRMS (EI) calcd for $C_8H_8N_4$, 160.0749; found, 160.0752. The purity of the compound was >95% by high-performance liquid chromatography (HPLC).

*5-Fuoroquinazoline-2,4-diamine (**4c**).* This substance was prepared from 2,6-difluorobenzonitrile (**5b**), using the general method described for preparation of **4b**, and obtained as a colorless powder (89 mg, 77%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.49 (brs, 1 H), 4.85 (brs, 2 H), 5.89 (brs, 1 H), 6.71−6.84 (m, 1 H), 7.22 (d, J = 8.53 Hz, 1 H), 7.48 (td, J = 8.11, 6.60 Hz, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 99.91 (d, J_{C-F} = 9.7 Hz), 105.07 (d, J_{C-F} = 22.6 Hz), 120.47 (d, J_{C-F} = 3.2 Hz), 132.53 (d, J_{C-F} = 11.9 Hz), 155.25, 160.02 (d, J_{C-F} =

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Table 3. Major Pharmacokinetic Parameters of 4o in Rats Following a Single Oral Dose (20 mg/kg) and a Single Intravenous Dose (10 mg/kg)

	$AUC_{0\rightarrow\infty}$ (ng h/mL)	MRT (h)	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/mL)	CLz (L/h/kg)	Vz (L/kg)	F (%)
op	2225	7.2	5.0	2.0	249			69
iv	1559	1.8	1.3			6.56	11.7	

4.3 Hz), 160.63, 161.15. HRMS (EI) calcd for $C_8H_7N_4F$, 178.0655; found, 178.0654. The purity of the compound was >95% by HPLC.

*6-Methoxyquinazoline-2,4-diamine (**4d**).* A mixture of 2-bromo-5-fluorobenzonitrile (**5c**, 200 mg, 0.50 mmol) and sodium methoxide (41 mg, 0.75 mmol) in 3 mL of *N*,*N*-dimethylformamide (DMF) was stirred at room temperature for 3 h, diluted with water (40 mL), and extracted with DCM (3 × 40 mL). The extracts were dried over Na_2SO_4, concentrated in vacuo, and used in the next step without further purification. A solution of the intermediate in 3 mL of *N*-methylpyrrolidone (NMP), containing guanidine carbonate (90 mg, 0.50 mmol), K_2CO_3 (69 mg, 0.50 mmol), and CuI (95 mg, 0.50 mmol), was stirred at 155 °C for 5 h, cooled to room temperature, diluted with water (40 mL), and extracted with DCM (3 × 50 mL). The extracts were dried over Na_2SO_4 and concentrated in vacuo to give a residue that was subjected to silica gel chromatography (12:1 DCM/MeOH, 1% $NH_3{\cdot}H_2O$) to afford **4d** as a colorless powder (61 mg, 64% over two steps). ^{1}H NMR (400 MHz, MeOH-d_4): δ 3.85 (s, 3 H), 7.20−7.30 (m, 2 H), 7.36 (s, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 55.55, 103.69, 123.26, 125.69, 147.54, 153.09, 159.66, 161.97. HRMS (EI) calcd for $C_9H_{10}N_4O$, 190.0855; found, 190.0856. The purity of the compound was >95% by HPLC.

*6-Phenoxyquinazoline-2,4-diamine (**4e**).* A mixture of 2-bromo-5-fluorobenzonitrile (**5c**, 200 mg, 0.50 mmol), phenol (57 mg, 0.60 mmol), and K_2CO_3 (104 mg, 75 mmol) in 3 mL of DMF was stirred at 100 °C for 8 h, cooled to room temperature, diluted with water (40 mL), and extracted with DCM (3 × 40 mL). The extracts were dried over Na_2SO_4, concentrated in vacuo, and used in the next step without further purification. A solution of the intermediate in 3 mL of NMP, containing guanidine carbonate (90 mg, 0.50 mmol), K_2CO_3 (69 mg, 0.50 mmol), and CuI (95 mg, 0.50 mmol), was stirred at 155 °C for 5 h, cooled to room temperature, diluted with water (40 mL), and extracted with DCM (3 × 50 mL). The extracts were dried over Na_2SO_4 and concentrated in vacuo to give a residue that was subjected to silica gel chromatography (12:1 DCM/MeOH, 1% $NH_3{\cdot}H_2O$) to afford **4e** as a colorless powder (39 mg, 31% over two steps). ^{1}H NMR (300 MHz, DMSO-d_6): δ 5.93 (s, 2 H), 6.91 (d, J = 7.62 Hz, 2 H), 7.04 (t, J = 7.33 Hz, 1 H), 7.14−7.29 (m, 4 H), 7.33 (t, J = 7.92 Hz, 2 H), 7.75 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 110.48, 113.79, 116.82, 122.37, 126.18, 126.37, 129.90, 148.27, 149.75, 158.35, 160.58, 162.12. HRMS (EI) calcd for $C_{14}H_{12}N_4O$, 252.1011; found, 252.1009. The purity of the compound was >95% by HPLC.

*8-Methoxyquinazoline-2,4-diamine (**4f**).* Compound **4f** was prepared from 2-bromo-3-fluorobenzonitrile (**5d**) using the general method described for the preparation of **4d** and obtained as a colorless powder (38 mg, 40% over two steps). ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.78 (s, 3 H), 6.04 (brs, 2 H), 6.85−7.00 (m, 2 H), 7.20 (brs, 2 H), 7.49 (d, J = 7.92 Hz, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 55.24, 110.63, 111.48, 115.05, 119.40, 143.79, 152.45, 160.03, 162.45. HRMS (EI) calcd for $C_9H_{10}N_4O$, 190.0855; found, 190.0858. The purity of the compound was >95% by HPLC.

*8-Phenoxyquinazoline-2,4-diamine (**4g**).* Compound **4g** was using the general method described for the preparation of **4d** and obtained as a colorless powder (45 mg, 36% over two steps). ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.06 (d, J = 7.43 Hz, 2 H), 7.12−7.27 (m, 3 H), 7.41 (t, J = 7.63 Hz, 2 H), 7.91 (d, J = 7.04 Hz, 1 H), 8.40 (brs, 2 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 111.26, 118.49, 119.22, 121.76, 122.24, 123.82, 130.11, 146.19, 156.41, 156.94, 156.99, 162.72. HRMS (EI) calcd for $C_{14}H_{12}NO$, 252.1011; found, 252.1004. The purity of the compound was >95% by HPLC.

*7-Methoxyquinazoline-2,4-diamine (**4h**).* A mixture of 2-bromo-4-fluorobenzonitrile (**5e**, 200 mg, 0.50 mmol) and sodium methoxide (41 mg, 0.75 mmol) in 3 mL of tetrahydrofuran (THF) was stirred at 50 °C for 6 h, cooled to room temperature, diluted with water (40 mL), and extracted with DCM (3 × 40 mL). The extracts were dried over Na_2SO_4, concentrated in vacuo, and used in the next step without further purification. A solution of the intermediate in 3 mL of NMP, containing guanidine carbonate (90 mg, 0.50 mmol), K_2CO_3 (69 mg, 0.50 mmol), and CuI (95 mg, 0.50 mmol), was stirred at 155 °C for 5 h, cooled to room temperature, diluted with water (40 mL), and extracted with DCM (3 × 50 mL). The extracts were dried over Na_2SO_4 and concentrated in vacuo to give a residue that was subjected to silica gel chromatography (12:1 DCM/MeOH, 1% $NH_3{\cdot}H_2O$) to afford **4h** as a colorless powder (41 mg, 43% over two steps). ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.77 (s, 3 H), 5.84 (s, 2 H), 6.51−6.64 (m, 2 H), 7.06 (brs, 2 H), 7.83 (d, J = 8.80 Hz, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 55.05, 104.11, 104.63, 110.66, 125.04, 154.70, 161.22, 162.12, 162.60. HRMS (EI) calcd for $C_9H_{10}N_4O$, 190.0855; found, 190.0850. The purity of the compound was >95% by HPLC.

*7-Phenoxyquinazoline-2,4-diamine (**4i**).* A mixture of 2-bromo-4-fluorobenzonitrile (**5e**, 200 mg, 0.50 mmol), phenol (57 mg, 0.60 mmol), and K_2CO_3 (104 mg, 75 mmol) in 3 mL of DMF was stirred at 45 °C for 6 h, cooled to room temperature, diluted with water (40 mL), and extracted with DCM (3 × 40 mL). The extracts were dried over Na_2SO_4, concentrated in vacuo, and used in the next step without further purification. A solution of the intermediate in 3 mL of NMP, containing guanidine carbonate (90 mg, 0.50 mmol), K_2CO_3 (69 mg, 0.50 mmol), and CuI (95 mg, 0.50 mmol), was stirred at 155 °C for 5 h, cooled to room temperature, diluted with water (40 mL), and extracted with DCM (3 × 50 mL). The extracts were dried over Na_2SO_4 and concentrated in vacuo to give a residue that was subjected to silica gel chromatography (12:1 DCM/MeOH, 1% $NH_3{\cdot}H_2O$) to afford **4i** as a colorless powder (57 mg, 45% over two steps). ^{1}H NMR (300 MHz, DMSO-d_6): δ 5.94 (s, 2 H), 6.49 (d, J = 2.05 Hz, 1 H), 6.68 (dd, J = 8.94, 2.49 Hz, 1 H), 7.08 (d, J = 7.92 Hz, 2 H), 7.14−7.27 (m, 3 H), 7.42 (t, J = 7.92 Hz, 2 H), 7.95 (d, J = 9.09 Hz, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 106.34, 109.64, 111.77, 119.70, 124.22, 125.85, 130.11, 154.13, 155.51, 160.66, 161.19, 162.17. HRMS (EI) calcd for $C_{14}H_{12}N_4O$, 252.1011; found, 252.1005. The purity of the compound was >95% by HPLC.

*5-Methoxyquinazoline-2,4-diamine (**4a**).* A mixture of 2,6-difluorobenzonitrile (**5b**, 100 mg, 0.72 mmol) and sodium methoxide (58 mg, 1.1 mmol) in 3 mL of THF was stirred at room temperature for 6 h, diluted with water (40 mL), and extracted with DCM (3 × 40 mL). The extracts were dried over Na_2SO_4, concentrated in vacuo, and used in the next step without further purification. A solution of the intermediate in 3 mL of DMA, containing guanidine carbonate (130 mg, 0.72 mmol), was stirred at 140 °C for 8 h, cooled to room temperature, diluted with water (40 mL), and extracted with DCM (3 × 50 mL). The extracts were dried over Na_2SO_4 and concentrated in vacuo to give a residue that was subjected to silica gel chromatography (15:1 DCM/MeOH, 1% $NH_3{\cdot}H_2O$) to afford **4a** as a colorless powder (122 mg, 83% over two steps). ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.88 (s, 3 H), 5.89 (s, 2 H), 6.50 (d, J = 7.92 Hz, 1 H), 6.75 (d, J = 8.21 Hz, 1 H), 7.15 (brs, 1 H), 7.32 (brs, 1 H), 7.34 (t, J = 8.21 Hz, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 55.78, 100.73, 101.23, 116.90, 132.47, 154.94, 157.47, 160.66, 161.82. HRMS (EI) calcd for $C_9H_{10}N_4O$, 190.0855; found, 190.0859. The purity of the compound was >95% by HPLC.

*5-Phenoxyquinazoline-2,4-diamine (**4j**).* A mixture of 2,6-difluorobenzonitrile (**5b**, 100 mg, 0.72 mmol), phenol (101 mg, 1.08 mmol), and K_2CO_3 (149 mg, 1.08 mmol) in 3 mL of DMF was stirred at 45 °C for 6 h, cooled to room temperature, diluted with water (40 mL), and extracted with DCM (3 × 40 mL). The extracts were dried over Na_2SO_4, concentrated in vacuo, and used in the next step without

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further purification. A solution of the intermediate in DMA, containing guanidine carbonate (130 mg, 0.72 mmol), was stirred at 140 °C for 8 h, cooled to room temperature, diluted with water (40 mL), and extracted with DCM (3 × 50 mL). The extracts were dried over Na_2SO_4 and concentrated in vacuo to give a residue that was subjected to silica gel chromatography (15:1 DCM/MeOH, 1% $NH_3 \cdot H_2O$) to afford **4j** as a colorless powder (146 mg, 81% over two steps). ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.83 (brs, 2 H), 6.34 (dd, J = 7.98, 1.10 Hz, 1 H), 7.13 (t, J = 7.84 Hz, 3 H), 7.21−7.29 (m, 3 H), 7.36 (t, J = 8.25 Hz, 1 H), 7.43 (t, J = 7.84 Hz, 2 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 102.47, 107.09, 118.97, 120.15, 124.71, 130.18, 132.36, 154.96, 155.03, 155.45, 160.63, 161.34. HRMS (EI) calcd for $C_{14}H_{12}N_4O$, 252.1011; found, 252.1010. The purity of the compound was >95% by HPLC.

*5-Ethoxyquinazoline-2,4-diamine (**4k**).* A solution of ethanol (40 mg, 0.86 mmol) in dry THF (1 mL) was added to a cooled (0 °C) THF solution (2 mL) containing sodium hydride (80% oil dispersion, 32 mg, 1.08 mmol) suspended in under nitrogen atmosphere. A solution of 2,6-difluorobenzolitrile (**5b**, 100 mg, 0.72 mmol) in THF (2 mL) was added at 0 °C, and the resulting mixture was stirred for 6 h at room temperature, poured on to crushed ice−water, and extracted with DCM (3 × 40 mL). The extracts were dried over Na_2SO_4 and concentrated in vacuo to afford intermediate without further purification. The solution of the intermediate in DMA, containing guanidine carbonate (130 mg, 0.72 mmol), was stirred at 140 °C for 8 h, cooled to room temperature, diluted with water (40 mL), and extracted with DCM (3 × 50 mL). The extracts were dried over Na_2SO_4 and concentrated in vacuo to give a residue that was subjected to silica gel chromatography (15:1 DCM/MeOH, 1% $NH_3 \cdot H_2O$) to afford **4k** as a colorless powder (97 mg, 66% over two steps). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.41 (t, J = 6.89 Hz, 3 H), 4.17 (q, J = 7.13 Hz, 2 H), 6.07 (s, 2 H), 6.55 (d, J = 8.21 Hz, 1 H), 6.77 (d, J = 8.21 Hz, 1 H), 7.34 (s, 2 H), 7.36 (t, J = 8.21 Hz, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.43, 64.40, 101.09, 102.08, 116.06, 132.87, 153.61, 156.60, 159.96, 161.97. HRMS (EI) calcd for $C_{10}H_{17}N_4O$, 204.1011; found, 204.1007. The purity of the compound was >95% by HPLC.

*5-Butoxyquinazoline-2,4-diamine (**4l**).* Compound **4l** was obtained from *n*-butanol using the general method described for the preparation of **4k** as a colorless powder (155 mg, 93% over two steps). ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.94 (t, J = 7.33 Hz, 3 H), 1.37−1.51 (m, 2 H), 1.73−1.85 (m, 2 H), 4.11 (t, J = 6.16 Hz, 2 H), 5.99 (s, 2 H), 6.54 (d, J = 7.33 Hz, 1 H), 6.76 (d, J = 7.92 Hz, 1 H), 7.25 (s, 2 H), 7.34 (t, J = 7.92 Hz, 1 H). ^{13}C NMR (100 MHz, DMSO- d_6): δ 13.66, 18.89, 30.43, 68.38, 101.08, 102.14, 116.05, 132.88, 153.59, 156.70, 159.90, 161.96. HRMS (EI) calcd for $C_{12}H_{16}N_4O$, 232.1324; found, 232.1321. The purity of the compound was >95% by HPLC.

*5-Octoxyquinazoline-2,4-diamine (**4m**).* Compound **4m** was obtained from *n*-octanol using the general method described for the preparation of **4k** as a colorless powder (143 mg, 69% over two steps). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.89 (t, J = 7.33 Hz, 3 H), 1.26−1.42 (m, 8H), 1.43−1.57 (m, 2 H), 1.84−1.96 (m, 2 H), 4.12 (t, J = 6.60 Hz, 2 H), 4.93 (brs, 2 H), 6.53 (d, J = 8.21 Hz, 1 H), 7.02 (d, J = 8.50 Hz, 1 H), 7.44 (t, J = 8.21 Hz, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 13.94, 22.06, 25.58, 28.31, 28.63, 28.66, 31.20, 68.72, 100.96, 102.55, 115.44, 133.17, 152.48, 156.72, 159.45, 162.00. HRMS (EI) calcd for $C_{16}H_{24}N_4O$, 288.1950; found, 288.1954. The purity of the compound was >95% by HPLC.

*5-(2,2,2-Trifluoroethoxy)quinazoline-2,4-diamine (**4n**).* Compound **4n** was obtained from 2,2,2-trifluoromethanol using the general method described for the preparation of **4k** as a colorless powder (102 mg, 55% over two steps). ^{1}H NMR (300 MHz, DMSO-d_6): δ 4.97 (q, J = 8.70 Hz, 2 H), 6.23 (brs, 2 H), 6.68 (d, J = 7.92 Hz, 1 H), 6.88 (d, J = 8.21 Hz, 1 H), 6.96 (brs, 1 H), 7.42 (t, J = 8.21 Hz, 1 H), 7.52 (brs, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 65.01 (q, J_{C-F} = 34.3 Hz), 100.91, 102.93, 117.58, 123.93 (q, J_{C-F} = 247.9 Hz), 132.76, 153.34, 154.62, 159.90, 161.47. HRMS (EI) calcd for $C_{10}H_9N_4OF_3$, 258.0728; found, 258.0730. The purity of the compound was >95% by HPLC.

*5-tert-Butoxyquinazoline-2,4-diamine (**4o**).* Compound **4o** was obtained from *tert*-butanol using the general method described for the preparation of **4k** as a colorless powder (127 mg, 76% over 2 steps). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.53 (s, 9 H), 4.78 (brs, 2 H), 5.60 (brs, 1 H), 6.75 (d, J = 7.70 Hz, 1 H), 7.07 (d, J = 7.98 Hz, 1 H), 7.40 (t, J = 8.11 Hz, 1 H), 7.74 (brs, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 28.45, 81.59, 104.62, 110.56, 117.92, 132.04, 153.60, 154.09, 159.95, 162.00. HRMS (EI) calcd for $C_{12}H_{16}N_4O$, 232.1324; found, 232.1326. The purity of the compound was >95% by HPLC.

*5-(Neopentyloxy)quinazoline-2,4-diamine (**4p**).* Compound **4p** was obtained from neopentanol using the general method described for the preparation of **4k** as a colorless powder (141 mg, 80% over two steps). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.12 (s, 9 H), 3.79 (s, 2 H), 4.87 (brs, 2 H), 6.53 (d, J = 7.98 Hz, 1 H), 7.03 (d, J = 8.53 Hz, 1 H), 7.44 (t, J = 8.25 Hz, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 26.55, 31.49, 78.50, 101.20, 101.83, 116.83, 132.68, 154.56, 156.92, 160.43, 161.94. HRMS (EI) calcd for $C_{13}H_{18}N_4O$, 246.1481; found, 246.1479. The purity of the compound was >95% by HPLC.

*5-(Cyclopentyloxy)quinazoline-2,4-diamine (**4q**).* Compound **4q** was obtained from cyclopentanol using the general method described for the preparation of **4k** as a colorless powder (136 mg, 77% over two steps). ^{1}H NMR (300 MHz, MeOH-d_4): δ 1.68−1.87 (m, 4 H), 1.87−2.14 (m, 4 H), 4.98−5.09 (m, 1 H), 6.67 (d, J = 8.25 Hz, 1 H), 6.86 (d, J = 8.25 Hz, 1 H), 7.45 (t, J = 8.25 Hz, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 23.56, 32.28, 80.31, 101.64, 103.12, 116.26, 132.58, 154.45, 155.45, 160.21, 161.98. HRMS (EI) calcd for $C_{13}H_{16}N_4O$, 244.1324; found, 244.1328. The purity of the compound was >95% by HPLC.

*5-(Cyclohexyloxy)quinazoline-2,4-diamine (**4r**).* Compound **4r** was obtained from cyclohexanol using the general method described for the preparation of **4k** as a colorless powder (178 mg, 96% over two steps). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.32−1.54 (m, 3 H), 1.55−1.72 (m, 3 H), 1.73−1.89 (m, 2 H), 2.05−2.17 (m, 2 H), 4.42−4.56 (m, J = 8.97, 8.97, 4.47, 4.26 Hz, 1 H), 5.22 (brs, 2 H), 5.68 (brs, 1 H), 6.57 (d, J = 8.25 Hz, 1 H), 7.01 (d, J = 8.25 Hz, 1 H), 7.43 (t, J = 8.25 Hz, 1 H), 7.77 (s, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 23.07, 24.94, 30.90, 75.77, 101.76, 103.29, 115.89, 132.76, 153.93, 155.29, 159.98, 162.06. HRMS (EI) calcd for $C_{14}H_{18}N_4O$, 258.1481; found, 258.1480. The purity of the compound was >95% by HPLC.

*5-(Benzyloxy)quinazoline-2,4-diamine (**4s**).* Compound **4s** was obtained from penylmethanol using the general method described for the preparation of **4k** as a colorless powder (88 mg, 46% over two steps). ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.17 (s, 2 H), 5.58 (brs, 2 H), 5.94 (brs, 1 H), 6.66 (d, J = 8.25 Hz, 1 H), 7.07 (d, J = 8.53 Hz, 1 H), 7.34−7.52 (m, 6 H), 7.59 (brs, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 70.29, 101.10, 103.19, 115.54, 128.05, 128.30, 128.72, 133.12, 136.25, 152.33, 156.35, 159.40, 161.93. HRMS (EI) calcd for $C_{15}H_{14}N_4O$, 266.1168; found, 266.1171. The purity of the compound was >95% by HPLC.

*N^5-tert-Butylquinazoline-2,4,5-triamine (**4t**).* To a thick-wall borosilicate glass vial (10 mL), 2,6-difluorobenzonirile (**5b**, 500 mg, 3.6 mmol), *tert*-butylamine (315 mg, 4.3 mmol), K_2CO_3 (745 mg, 5.4 mmol), and DMSO (2 mL) were added. The reaction vial was sealed and placed in the microwave reactor and irradiated as at 110 °C for 15 min. After it was cooled to room temperature, the mixture was extracted with EtOAc, washed by saturated brine, and dried over Na_2SO_4. The filtrate was concentrated in vacuo giving a residue that was subjected to silica gel chromatography [petroleum ether (PE) to 7% EtOAc in PE] to afford intermediate **7a** (522 mg, 76%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.43 (s, 9 H), 4.65 (brs, 1 H), 6.37 (t, J = 8.39 Hz, 1 H), 6.67 (d, J = 8.53 Hz, 1 H), 7.21−7.33 (m, 1 H).

A solution of **7a** (100 mg, 0.52 mmol) and guanidine carbonate (94 mg, 0.52 mmol) in 3 mL of DMA was stirred at 140 °C for 8 h, cooled to room temperature, diluted with water (40 mL), and extracted with DCM (3 × 50 mL). The extracts were dried over Na_2SO_4 and concentrated in vacuo to give a residue that was subjected to silica gel chromatography (12:1 DCM/MeOH, 1% $NH_3 \cdot H_2O$) to afford **4t** as a colorless powder (94 mg, 78%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.08 (s, 9 H), 4.52 (brs, 1 H) 5.88 (s, 2 H), 6.63 (d, J = 7.62 Hz, 1 H), 6.93 (d, J = 8.21 Hz, 1 H), 7.30 (t, J = 7.92 Hz, 1 H), 8.34 (brs, 2 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 28.41, 53.65, 109.22, 120.21, 120.53, 130.96, 143.42, 153.36, 159.68, 163.39. HRMS (EI) calcd for

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$C_{12}H_{17}N_5$, 231.1484; found, 231.1483. The purity of the compound was >95% by HPLC.

*N^5,N^5-Dimethylquinazoline-2,4,5-triamine (**4u**).* A mixture of 2,6-difluorobenzonitrile (**5b**, 500 mg, 3.6 mmol), dimethylamine hydrochloride (352 mg, 4.3 mmol), and K_2CO_3 (745 mg, 5.4 mmol) in DMSO (2 mL) was added to a thick-wall borosilicate glass vial (10 mL). Then, the reaction vial was sealed and placed in the microwave reactor and irradiated as at 110 °C for 15 min. After it was cooled to room temperature, the mixture was extracted with EtOAc, washed by saturated brine, and dried over Na_2SO_4. The filtrate was concentrated in vacuo giving a residue that was subjected to silica gel chromatography (PE to 7% EtOAc in PE) to afford intermediate **7b** (372 mg, 63%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.10 (s, 6 H), 6.48–6.65 (m, 2 H), 7.32 (td, J = 8.39, 6.88 Hz, 1 H).

A solution of **7b** (100 mg, 0.61 mmol) and guanidine carbonate (110 mg, 0.61 mmol) in 3 mL of DMA was stirred at 140 °C for 8 h, cooled to room temperature, diluted with water (40 mL), and extracted with DCM (3 × 50 mL). The extracts were dried over Na_2SO_4 and concentrated in vacuo to give a residue that was subjected to by silica gel chromatography (12:1 DCM/MeOH, 1% $NH_3{\cdot}H_2O$) to afford **4u** as a colorless powder (62 mg, 50%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.63 (s, 6 H) 6.02 (s, 2 H), 6.86 (d, J = 7.62 Hz, 1 H), 6.93 (d, J = 8.21 Hz, 1 H), 7.32 (brs, 1 H), 7.38 (t, J = 8.06 Hz, 1 H), 8.89 (brs, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 45.38, 105.54, 111.10, 120.56, 132.04, 152.67, 154.38, 160.14, 162.17. HRMS (EI) calcd for $C_{10}H_{13}N_5$, 203.1171; found, 203.1168. The purity of the compound was >95% by HPLC.

*5-(Pyrrolidin-1-yl)quinazoline-2,4-diamine (**4v**).* Compound **4v** was obtained from pyrrolidine using the general method described for the preparation of **4u** as a colorless powder (64 mg, 53% over two steps). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.73–2.02 (m, 4 H), 2.60–2.88 (m, 2 H), 3.09–3.26 (m, 2 H), 5.87 (s, 2 H), 6.82 (dd, J = 7.77, 1.03 Hz, 1 H), 6.90 (dd, J = 8.21, 1.17 Hz, 1 H), 7.10 (brs, 1 H), 7.34 (t, J = 7.92 Hz, 1 H), 8.56 (brs, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 26.81, 56.40, 109.27, 113.98, 122.94, 135.46, 152.01, 156.93, 163.00, 165.54. HRMS (EI) calcd for $C_{12}H_{15}N_5$, 229.1327; found, 229.1330. The purity of the compound was >95% by HPLC.

*5-(tert-Butylthio)quinazoline-2,4-diamine (**4w**).* A mixture of 2,6-difluorobenzonitrile (**5b**, 250 mg, 1.8 mmol), *tert*-butylthiol (178 mg, 2.0 mmol), and K_2CO_3 (298 mg, 2.2 mmol) in 4 mL of DMF was stirred at room temperature for 12 h, diluted with water (40 mL), and extracted with DCM (3 × 40 mL). The extracts were dried over Na_2SO_4, concentrated in vacuo, and used in the next step without further purification. A solution of the intermediate and guanidine carbonate (324 mg, 1.8 mmol) in 4 mL of DMA was stirred at 140 °C for 8 h, cooled to room temperature, diluted with water (40 mL), and extracted with DCM (3 × 50 mL). The extracts were dried over Na_2SO_4 and concentrated in vacuo to give a residue that subjected to silica gel chromatography (15:1 DCM/MeOH, 1% $NH_3{\cdot}H_2O$) to afford **4w** as a colorless powder (438 mg, 98%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.22 (s, 9 H), 6.04 (s, 2 H), 7.16 (d, J = 7.04 Hz, 1 H), 7.28 (d, J = 8.21 Hz, 1 H), 7.41 (brs, 1 H), 7.43 (t, J = 8.06 Hz, 1 H), 8.72 (brs, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 30.08, 48.75, 112.47, 127.40, 127.59, 131.04, 133.92, 154.61, 159.82, 162.56. HRMS (EI) calcd for $C_{12}H_{16}N_4S$, 248.1096; found, 248.1100. The purity of the compound was >95% by HPLC.

*5-(tert-Butylsulfonyl)quinazoline-2,4-diamine (**4x**).* A solution of Oxone (990 mg, 1.6 mmol) and **4w** (100 mg, 0.40 mmol) in 4 mL of MeOH and 2 mL of water was stirred at room temperature stirred for 12 h, diluted with water (40 mL), and extracted with DCM (3 × 40 mL). The extracts were dried over Na_2SO_4 and concentrated in vacuo give a residue that was subjected to silica gel chromatography (10:1 DCM/MeOH, 1% $NH_3{\cdot}H_2O$) to afford **4x** as a yellow powder (76 mg, 67%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.21 (s, 9H), 7.58 (brs, 2 H), 7.72–7.78 (m, 2 H), 7.95 (dd, J = 6.45, 2.64 Hz, 1 H), 8.48 (brs, 1 H), 9.58 (brs, 1 H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 26.02, 66.27, 109.78, 131.92, 132.39, 134.56, 136.88, 147.82, 151.70, 153.39. HRMS (EI) calcd for $C_{12}H_{16}N_4O_2S$, 280.0994; found, 280.0993. The purity of the compound was >95% by HPLC.

DENV-2 Replicon Cell Line. A DENV-2 replicon carrying a firefly luciferase gene as a reporter was constructed using an infectious full length DENV-2 plasmid pD2FT, which was derived from the DENV-2 (New Guinea C strain) cDNA. Initially, the structure protein coding sequence of CprME was deleted by using the PCR method. A Luc-IRES-APH gene cassette was constructed in a pMD18 vector. The gene cassette was then amplified by using PCR and ligated with pD2FT PCR, which lacked a structure gene coding sequence, to form a DENV-2 replicon plasmid driven by T7 protomer named pD2RepT. The replicon genome diagram is shown in Figure 4. The plasmid was

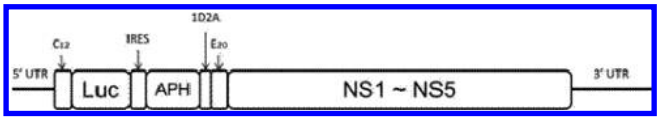

Figure 4. Replicon genome diagram.

linearized by using Xba I, and replicon RNA was transcribed using a Ribomax T7 RNA synthesis kit (Promega) in the presence of cap analogue m7GpppA (NEB). Replicon RNA was then transfected into BHK cell using Lipofectamin 2000 reagent. Cells were cultured in MEM medium containing 10% FBS and 400 μg/mL G418. Resistant clones expressing the highest amounts of luciferase and viral non-structural proteins NS3 were selected and used as a DENV-2 replicon cell line named BHK-D2RepT. Cells were propagated in MEM medium containing 10% FBS and 200 μg/mL G418.

Antiviral Reporter Assay. The BHK-D2RepT cell was preseeded in 96-well white plates (Costar) for 24 h without G418. The substance was dissolved in DMSO as stocking solution, diluted with MEM medium to reach the desired concentration, and added to the cell culture medium. Final concentrations of DMSO in the culture medium were less than 0.2% (v/v). After the substance was added, the cells were cultured for 24 h. The culture medium was replaced by fresh MEM medium, and the luciferase signal was assayed by using a Bright-Glo firefly luciferase assay kit (Promega). The inhibitory rate was calculated as the percentage of luciferase signals as compared to that produced when no substance was present.

Infectious DENV-2 Virus Test. Infectious DENV-2 virus (NGC strain) was propagated in C6/36 cells. The viral supernatant was collected and titered. For antiviral tests in BHK cells, cells were preseeded in a 6-well plate overnight and infected with DENV-2 virus at MOI = 1 for 2 h. Multiplicity of infection (MOI) is the ratio of infectious virus particles versus cell number; therefore, MOI = 1 means nearly all cells were infected by virus initially, thus producing one round of virus replication cycle in the cell culture with no second round infection by progeny virus. Virus inoculation was replaced with fresh medium after initial infection, and the substances were added to the desired concentrations. After 48 h, cells were trypsinized and fixed for viral NS1 antigen staining by using the indirect immunofluorescence method and detected by using flowcytometry.

In the C6/36 cell antiviral test, C6/36 cells were preseeded overnight before virus infection at MOI = 0.01. In this case, several rounds of the life cycle of the virus took place to produce syncytia of C6/36 cells. The substance was added and further cultured for 4 days. The virus-induced cytopathtic effect was observed by using optical microscopy.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details for intermediates and target compounds and HPLC purity data for target compounds **4a–x**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

EC_{50}, half maximal effective concentration; CC_{50}, 50% cytotoxicity concentration; DMA, *N*,*N*-dimethylacetamide; DMF, *N*,*N*-dimethylformamide; THF, tetrahydrofuran; DCM, dichloromethane; MeCN, acetonitrile; PE, petroleum ether; NMP, *N*-methylpyrrolidone; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectrum; NMR, nuclear magnetic resonance; iv, Intravenous injection; po, oral administration; AUC, the area under the plasma concentration time curve; MRT, mean residence time; $t_{1/2}$, half-life; T_{max}, time to peak concentration; C_{max}, peak concentration; CLz, clearance; Vz, terminal volume of distribution; *F*, oral bioavailability

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