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Anti-Ebola Activity of Diazachrysene Small Molecules

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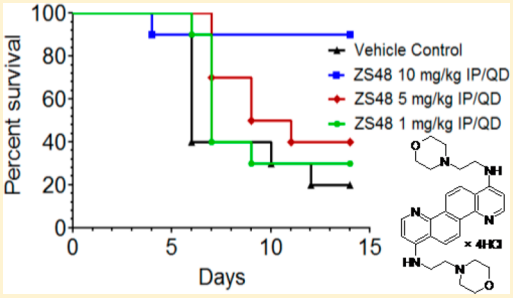
Supporting Information

ABSTRACT: Herein we report on a diazachrysene class of small molecules that exhibit potent antiviral activity against the Ebola (EBOV) virus. The antiviral compounds are easily synthesized, and the most active compounds have excellent in vitro activity (0.34−0.70 μM) and are significantly less lipophilic than their predecessors. The three most potent diazachrysene antivirals do not exhibit any toxicity in vivo and protected 70−90% of the mice at 10 mg/kg following EBOV challenge. Together, these studies suggest that diazachrysenes are a promising class of compounds for hit to lead optimization and as potential Ebola therapeutics.



KEYWORDS: *Ebola virus, antiviral diazachrysenes, inhibitory efficacy small molecule, toxicity*

The Ebola virus (EBOV) belongs to the *Filoviridae* family of single-stranded, negative sense RNA viruses and causes the deadly hemorrhagic fever in humans and nonhuman primates.[1,2] Filoviruses are highly infectious and lethal, and to date there are no FDA-approved therapeutics to treat or vaccines to protect against EBOV infections. Therefore, the United States Centers for Disease Control and Prevention classifies filoviruses as category A bioterrorism agents. The ongoing Ebola epidemic in West Africa has affected >23 500 people with >9600 deaths,[3] and the swift rate at which the infection proceeds leaves little opportunity for acquired immunity to develop.[4] Among several promising vaccines, at present two candidate platforms are going through phase 1 prelicensure clinical studies.[5] The occasional occurrence and rapid progression of filovirus diseases emphasizes the need to create small molecules that will provide immediate therapeutic benefit.

The development of experimental therapeutics may include those that target viral proteins, host pathways, and direct-acting antivirals. A number of small molecules and large-protein-based biologics have been evaluated and shown to possess antifilovirus activity in vitro and/or in vivo.[6] Screening of the FDA-approved drug library in Ebola virus infection assays identified several drugs that exhibited anti-Ebola virus activity.[7,8] These screening campaigns identified several interesting small-molecule inhibitors that were intended for use against other disease indications. Interesting findings include antimalarial chloroquine that protected mice against EBOV challenge.[7] In addition, the in vitro activity of the other small-molecule classes was thoroughly reviewed for its anti-EBOV activity recently.[9,10] New small molecules such as adenosine analog BCX4430,[11] brincidofovir,[12] and favipiravir[13] are the most promising new entities under development in clinical evaluation. Inhibitors with mechanisms of action that are unclear at present[14] include 4-quinoline carboxylic acid analogues[15] as well as inhibitors of filoviruses that target host−cell protein interaction.[16]

Our own previously published studies on small molecules inhibiting Ebola virus infection comprise the antioxidants,[17] diazachrysenes,[18−20] and adenosine analogs.[11] It was shown that diazachrysenes are good nontoxic small-molecule inhibitors of botulinum neurotoxic A light chain and antiplasmodials with interesting in vitro activity.[18] Moreover, tested diazachrysenes showed favorable metabolic stability (HLM, MLM, ∼60 min), low toxicity in vitro, and no toxicity in vivo.[18] The analysis of physicochemical properties of diazachrysenes[21] indicated that the compounds are in the form of a BH_3^{3+} cation at physiologically pH 7.4; however, the relative lipophilicity of compounds **2** and **3** (Chart 1) might unfavorably influence their activity. For that purpose, we devised the synthesis of des-methyl compounds and indeed discovered that des-methyl diazachrysene **5** (Chart 1) has significantly more potent anti-EBOV activity in in vitro studies in comparison to its predecessor, **2**.[19] Here we report on the synthesis and the in vitro and in vivo screening results of compound **5** and newly synthesized EBOV inhibitors as HCl salts **5** and **16**−**23** (Scheme 1).

RESULTS AND DISCUSSION

Chemistry. For the amination of starting dichloro-diazachrysene **6**,[19] the microwave conditions were developed

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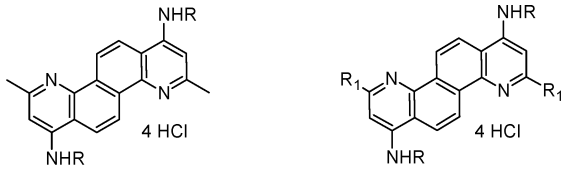
Chart 1. Structures of Potent Pyrrolidino and Morpholino EBOV Inhibitors[18,19]



1: R = $CH_2CH_2CH_2N(CH_2)_4$
97% inh. at 20μM
IC_{50} = 5-10 μM[18]

2: R = $CH_2CH_2N(CH_2CH_2)O$
53% inh. at 20μM[18]

3: R = $CH_2CH_2CH_2N(CH_2CH_2)_2O$
96% inh. at 20μM
IC_{50} = 2.5-5 μM[18]

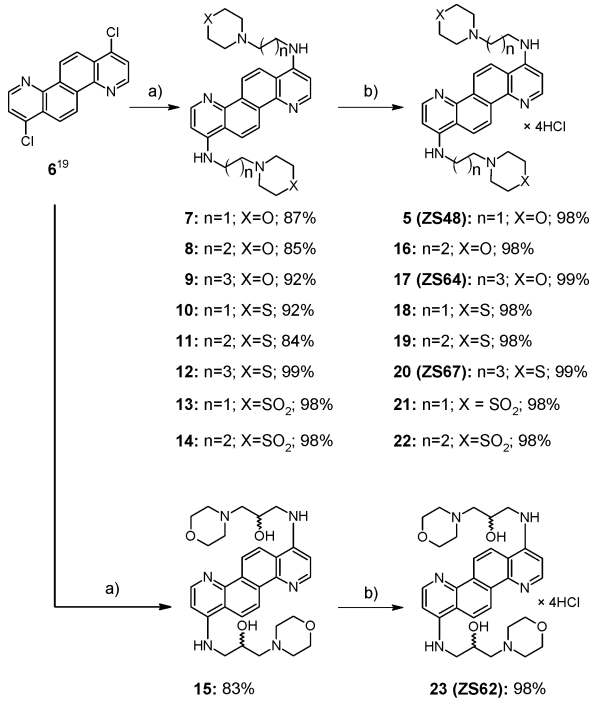
4: R = $CH_2CH_2N(CH_2CH_2)_2O$

R_1 =

IC_{50} = 12.98 μM[19]

5: R = $CH_2CH_2N(CH_2CH_2)_2O$
R_1 = H; 100% inh. at 20μM
IC_{50} = 0.70 μM[19]

Scheme 1. Synthesis of Diazachrysene Salts 5 and 16−23



6[19]

7: n=1; X=O; 87%
8: n=2; X=O; 85%
9: n=3; X=O; 92%
10: n=1; X=S; 92%
11: n=2; X=S; 84%
12: n=3; X=S; 99%
13: n=1; X=SO_2; 98%
14: n=2; X=SO_2; 98%

5 (ZS48): n=1; X=O; 98%
16: n=2; X=O; 98%
17 (ZS64): n=3; X=O; 99%
18: n=1; X=S; 98%
19: n=2; X=S; 98%
20 (ZS67): n=3; X=S; 99%
21: n=1; X = SO_2; 98%
22: n=2; X=SO_2; 98%

15: 83%

23 (ZS62): 98%

a) amine, MW, NMP, 180 °C, 6 h, Ar; b) HCl/MeOH, 1 h, r.t.

that enabled the use of considerably less amine than in previous syntheses, as illustrated in Table 1.[18] Commercially available 2-(morpholin-4-yl)ethanamine was used for optimization experiments.

After successful optimization, **6** and the appropriate amine were held at 180 °C in an MW reactor in NMP solvent for 6 h before quenching the reaction (Scheme 1). The appropriate amines were used as purchased or were synthesized as described in the Supporting Information.

Nucleophilic substitution on the diazachrysene entity under MW conditions yielded respective diamino derivatives **7**−**15** in 83−99% yield, and crude products were further transformed into their water-soluble HCl salts **5** and **16**−**23**.

Anti-EBOV Activity. Preliminary screening of compounds **5** and **16**−**23** helped determine the cellular efficacy as well as associated cytotoxicity during the course of EBOV infection. All diazachrysenes were tested as HCl salts. HeLa cells were pretreated with the compounds **5** and **16**−**23** (20, 10, 5 μM) for 2 h and subsequently infected with EBOV Zaire-95 (MOI = 0.5) for 48 h. Ebola-infected cells were detected using anti-GP antibody and quantitated using our previously published high-content image analysis method.[22] A total of 9 compounds out of the 11 that were tested exhibited 50−100% anti-Ebola activity (Table 2). Diazachrysene **18** inhibited EBOV infection by 50%, and sulfones **21** and **22** were inactive. Of the six most active inhibitors, **16** and **19** exhibited high cellular toxicity as measured by ≥95% reduction in HeLa host cell number, and they were discarded from further evaluation.

Next, dose−response studies were conducted to systematically inspect the efficacies and toxicities of **5**, **23**, **17**, and **20** at 10 concentrations during EBOV cellular infection (10 points with a 2-fold dilution step). Each compound was tested in four replicates (n = 4), and the experiment was repeated on two independent days. The EC_{50} values of the compounds are shown in Table 2, and dose−response curves for representative compounds **17** (**ZS64**), **20** (**ZS67**), and **5** (**ZS48**) are shown in Figure 1. The most active diazachrysene was morpholino derivative **17** (**ZS64**) having a C4 linker with EC_{50} = 340 nM. Thiomorpholino derivative **20** (**ZS67**) also possessing a C4 linker had a similar EC_{50} value of 360 nM (Table 2). None of

Table 1. Optimization of Amination Reaction Conditions

experiment	amine (equiv)	solvent	temp (°C)	reaction time (h)	7 (%)[a]
1	30	none	180	60	81
2	6	NMP	180	6	11
3	6	NMP	MW, 180	6	87

[a]Yield of isolated product.

Table 2. Inhibitory Activity of Diazachrysenes against Ebola, log P_{ow}, and in Vivo Mice Survival

compound	% EBOV inhibition, (% reduction of cell number), 20 μM[a]	EBOV EC_{50}, μM	log P_{ow} exp	% mice survival 10 mg/kg, IP[b]
2	53[18]		3.88[21]	
3	96[18]	2.5−5	4.24[21]	
5 (ZS48)	100 (0)	0.70 ± 0.13[19]	1.83	90
16	100 (95)	2.5−5	2.12	
17 (ZS64)	100 (2) at 10 μM	0.34 ± 0.01	2.27	70
18	50 (0)	20	3.23	
19	100 (99)	0.625−1.25	3.37	
20 (ZS67)	100 (0) at 5 μM	0.36 ± 0.06	3.51	20
21	not active at 10 μM	>10 μM	1.83	
22	not active at 10 μM	>10 μM	1.98	
23 (ZS62)	88 (0)	1.25−2.5	1.39	70
BCX4430[11]		11.8		100 (IM 150 mg/kg)
NSC62914[17]		5−10		50 (2 mg/kg)
CQ[7]		4.7		80 (90 mg/kg)

[a]Reduction of host HeLa cells. [b]10 mg/kg. Mice (n = 10 mice/group) were treated via the IP route and after 2 h were challenged via the IP route with 1000 pfu mouse-adapted EBOV. Treatment was continued once daily for 6 days.

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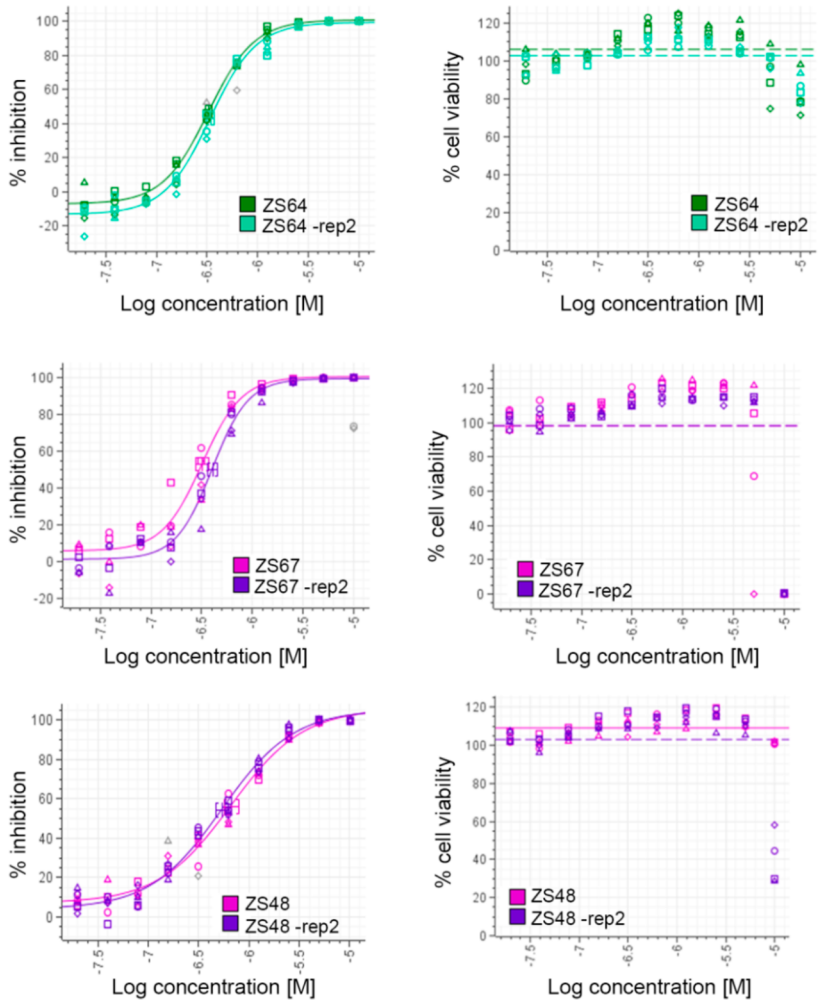

Figure 1. Dose-dependent graphs for compounds **17** (**ZS64**), **20** (**ZS67**), and **5** (**ZS48**). Dose-dependent inhibition of EBOV infection in HeLa cells pretreated with the compounds for 2 h and then infected with the virus for 48 h. Data was analyzed using Genedata software. Graphs of % EBOV inhibition (left panel) and % cell viability based on loss of cell number (right panel) for compounds **17** (**ZS64**), **20** (**ZS67**), and **5** (**ZS48**) are depicted.

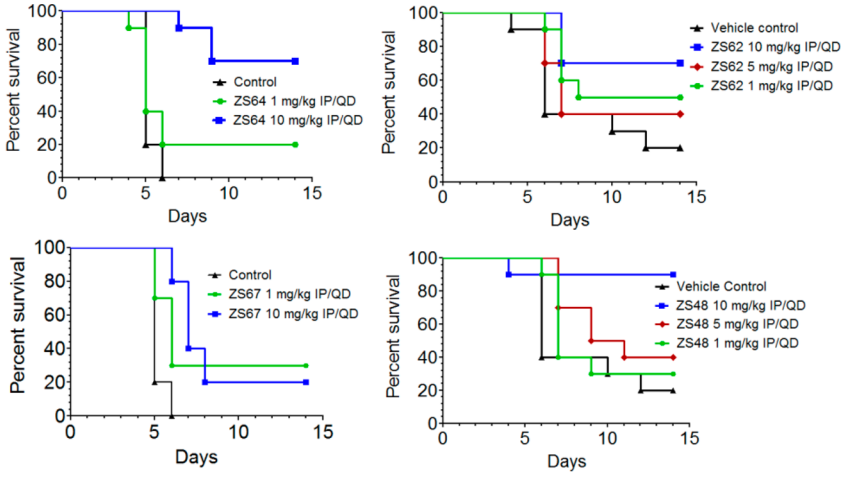

Figure 2. Diazachrysenes **5** (**ZS48**), **17** (**ZS64**), **20** (**ZS67**), and **23** (**ZS62**) protect mice from EBOV challenge. Mice (n = 10 mice/group) were treated with specified concentrations of the compounds via the IP route and after 2 h challenged via the IP route with 1000 pfu of mouse-adapted EBOV. Treatment was continued once daily for 6 days. All p values are adjusted by Dunnett's method to account for multiple comparisons. **5** (**ZS64**), $p < 0.0001$ (10 mg/kg); **17** (**ZS67**), $p = 0.0017$ (10 mg/kg); **20** (**ZS62**), $p = 0.0247$ (10 mg/kg); **23** (**ZS48**), $p = 0.0029$ (10 mg/kg).

the four compounds (**5**, **17**, **20**, or **23**) exhibited any associated toxicity with respect to the host cells.

In Vivo Efficacy Studies. The most promising and nontoxic in vitro inhibitors **5**, **17**, **20**, and **23** were further

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evaluated for efficacy in the mouse model of Ebola infection. We used the mouse-adapted Ebola Zaire virus (ZEBOV), and four groups of 10 Balb/c mice were infected via the IP route with ZEBOV (1000 pfu). The mice were IP treated with respective inhibitor concentrations with a daily single dose (QD) from days 0−7. For details, see the Methods section.

Compounds **5** (**ZS48**) and **23** (**ZS62**) were tested at three concentrations, of 10, 5, and 1 mg/kg in PBS-DMSO as a solvent (control). Compounds **17** (**ZS64**) and **20** (**ZS67**) were soluble in water and were administered via the IP route at concentrations of 10 and 1 mg/kg (Figure 2).

DISCUSSION

Here we present the synthesis of nine new diazachrysenes and report their in vitro and in vivo activity against the Ebola virus. The compounds were easily prepared in two steps starting from known dichlorodiazachrysene **6**[19] in a 81−91% overall yield. All compounds were prepared and tested as HCl salts (Scheme 1); they were fully characterized and were >95% pure (Methods section and Supporting Information). This series of diazachrysene EBOV inhibitors was prepared as a logical consequence of our previous results on antiviral characteristics of their predecessors.[18,19] In addition, we have shown that tested diazachrysenes are nontoxic and are metabolically relatively stable.[18] The current diazachrysene series was also found to be nontoxic in vitro, and in mouse challenge studies the administered inhibitors were well tolerated and no apparent clinical manifestations of toxicity were produced. This was followed by observations of individual mouse behavior and appearance two times a day.[23]

A series of diazachrysenes possessing methyl groups in positions C3 and C9 were analyzed in detail for their physicochemical properties.[21] We found that lipophilicity quantified as experimentally determined partition coefficients log P_{ow} was relatively high (3.51−5.80). The most interesting compounds, **2** and **3** (Table 1), appeared to be relatively lipophilic with log P_{ow} = 3.88 and 4.24, respectively.[21] With that in mind we decided to strip off the methyl groups from position C3 and C9. New inhibitors were synthesized according to Scheme 1, and log P_{ow} values of new compounds were experimentally determined. log P_{ow} of diazachrysene **5** appeared to be much lower than that of **2**, 1.83 vs 3.88; log P_{ow} of compound **16** was lower than that of **3**, 2.12 vs 4.24. In addition, we prepared hydroxy derivative **23** of parent compound **16** which, as expected, had an even lower log P_{ow} of 1.39.

The elimination of methyl groups from the aromatic core of diazachrysenes significantly enhanced the in vitro anti-EBOV activity of des-methyl compounds; e.g., diazachrysene **5** was 2 times more active than its predecessor **2**, with 100% inhibition vs 53% inhibition at 20 μM. Morpholino derivative **16** retained the excellent inhibitory activity of its predecessor **2**; however, it developed toxicity in vitro and hence was not further scrutinized. The EC_{50} values were determined from dose-dependent curves, and the two most active compounds in vitro were morpholino and thiomorpholino derivatives **17** (**ZS64**) and **20** (**ZS67**), both having a C4 linker. In the mouse challenge test, however, the two compounds behaved in a totally different manner. Namely, thiomorpholino derivative **20** (**ZS67**) did not provide a dose-dependent survival (Figure 2). Its oxygen analog, morpholino derivative **17** (**ZS64**), however, enabled a dose-dependent increase in survival with 7/10 mice protected at dose of 10 mg/kg and 2/10 mice protected at 1 mg/kg. Higher lipophilicity and the possible metabolic oxidation of sulfur atoms under in vivo conditions evidently played a role in the inferior activity of thiomorpholine **20** (**ZS67**) in comparison to its morpholine counterpart **17** (**ZS64**).

The two least lipophilic diazachrysenes with morpholine side chains **5** (**ZS48**) and **23** (**ZS62**) were tested in the Ebola infection model at concentrations of 10, 5, and 1 mg/kg. Compound **5** (**ZS48**) provided 90% survival at 10 mg/kg and dose-dependent protection at 5 and 1 mg/kg (40 and 30% survival, respectively). The other diazachrysene, **23** (**ZS62**), with its 2-hydroxypropylmorpholinyl side chain enabled 70% survival at 10 mg/kg concentration and inverse protection at 1 and 5 mg/day (50 and 40% survival, respectively). Evidently, the morpholino side chain was an excellent substituent for the robust diazachrysene body.

Together, these studies suggest that diazachrysenes are promising EBOV inhibitors. However, their mode of action needs to be investigated. Various surrogate systems such as the pseudotype, minigenome, and VLP assays will help to determine the steps in the viral infection process with which the compounds may be interfering. Alternatively, the compounds may be acting on host targets. Chemically linking these compounds with appropriate tags and subsequent pull down studies in combination with mass spectrometry may help to identify the potential target of these compounds. In addition, our current research is aimed at better delivery and improvements in PK/PD properties.

CONCLUSIONS

Here we report on the very promising class of small molecules that exhibit potent antiviral activity against the Ebola virus. The antiviral compounds are easily synthesized, have excellent in vitro activity, and are significantly less lipophilic than their predecessors. The proof of concept was obtained upon testing in vivo: our diazachrysenes are not toxic in vivo, and three active diazachrysene antivirals protected 70−90% of the mice at 10 mg/kg following EBOV challenge. These results confirm that this class of small molecules is suitable for further development as promising Ebola virus inhibitors.

METHODS

Chemistry. Melting points were determined on a Boetius PMHK apparatus and were not corrected. IR spectra were taken on a Thermo-Scientific Nicolet 6700 FT-IR diamond crystal. NMR: ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Ultrashield Advance III spectrometer (at 500 and 125 MHz, respectively) in the indicated solvent using TMS as the internal standard. Chemical shifts are expressed in ppm (δ) values, and coupling constants (J), in Hz. ESI MS spectra of the synthesized compounds were recorded on an Agilent Technologies 6210 time-of-flight LC/MS instrument in positive ion mode using CH_3CN/H_2O = 1/1 with 0.2% HCOOH as the carrying solvent solution. The samples were dissolved in pure acetonitrile (HPLC grade). The selected values were as follows: capillary voltage = 4 kV; gas temperature = 350 °C; drying gas = 12 L min^{-1}; nebulizer pressure = 45 psig; and fragmentator voltage = 70 V.

HPLC Purity Determination. Compounds **5** and **16**−**23** were analyzed for purity (HPLC) using a Waters 1525 HPLC dual-pump system equipped with an Alltech Select degasser system and a dual λ 2487 UV−vis detector and using an Agilent

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1200 HPLC system equipped with a Quat pump (G1311B), an injector (G1329B) 1260 ALS, TCC 1260 (G1316A), and a detector 1260 DAD VL+ (G1315C). All compounds were ≥95% pure. For details, see the Supporting Information.

General Procedure for the Preparation of 1,7-Bis-(alkylamino)-4,10-diazachrysenes. Dichlorodiazachrysene **6**[19] and an excess of the appropriate amine were dissolved in NMP in a MW cuvette under argon. The reaction mixture was subjected to MW irradiation using a Biotage Initiator 2.5 apparatus for 6 h at 180 °C. The cooled reaction mixture was poured onto ice−water. The obtained precipitate was filtered, washed with water, and dried under reduced pressure.

General Procedure for the Preparation of 1,7-Bis-(alkylamino)-4,10-diazachrysene Hydrochlorides. The appropriate base was suspended in 40% HCl in dry MeOH, and the reaction mixture was vigorously stirred for 1 h at r.t. The solvent was then removed under reduced pressure, and the remaining solid was suspended in dry EtOH. The EtOH was removed under reduced pressure, and the same procedure with EtOH was repeated two more times. The desired product was obtained upon drying at 40 °C under reduced pressure.

*N,N′-Bis[2-(morpholin-4-yl)ethyl]quinolino[8,7-h]-quinoline-1,7-diamine (**7**).*[19] The general procedure provided above was followed using **6** (35.4 mg, 0.118 mmol), 2-(morpholin-4-yl)ethanamine (160 mg, 1.23 mmol), and NMP (1 mL). The yield was 50.1 mg (87%). **7**: Light-brown powder, mp >280 °C. IR (ATR): 3436m, 3413w, 2962w, 2855m, 2813m, 1594s, 1534s, 1505w, 1456m, 1332w, 1275w, 1239w, 1178w, 1142m, 1118s, 1070w, 1030w, 952w, 911w, 862w, 822 w, 763w cm^{-1}. ^{1}H NMR (500 MHz, TFA): 8.97 (d, J = 9.4, 2H), 8.68 (d, J = 7.2, 2H), 8.58 (d, J = 9.4, 2H), 7.30 (d, J = 7.2, 2H), 4.40−4.35 (m, 8H), 4.11 (t, J = 12.4, 4H), 3.98−3.91 (m, 8H), 3.57−3.48 (m, 4H). ^{13}C NMR (125 MHz, TFA): 159.11, 144.79, 137.79, 127.84, 123.35, 123.27, 119.30, 103.69, 66.14, 57.79, 55.27, 40.13. HRMS: m/z 487.28029 corresponds to molecular formula $C_{28}H_{34}N_6O_2H^+$ (error in ppm −2.69).

*N,N′-Bis[3-(morpholin-4-yl)propyl]quinolino[8,7-h]-quinoline-1,7-diamine (**8**).* The general procedure provided above was followed using **6** (50.9 mg, 0.170 mmol), 3-(morpholin-4-yl)propanamine (283.6 mg, 1.967 mmol), and NMP (2 mL). The yield was 74.5 mg (85%). **8**: Light-brown powder, mp = 276−278 °C. IR (ATR): 3750w, 3400s, 2923m, 2852w, 1595s, 1541m, 1457w, 1431w, 1343m, 1243w, 1113m, 1005w, 862w, 804w, 756w, 546w cm^{-1}. ^{1}H NMR (500 MHz, TFA): 8.88 (d, J = 9.2, 2H), 8.62 (d, J = 7.2, 2H), 8.58 (d, J = 9.2, 2H), 7.19 (d, J = 7.4, 2H), 4.40−4.35 (m, 4H), 4.13−4.07 (m, 4H), 3.96−3.91 (m, 4H), 3.83−3.79 (m, 4H), 3.65−3.60 (m, 4H), 3.47−3.40 (m, 4H), 2.53−2.59 (m, 4H). ^{13}C NMR (125 MHz, TFA): 159.14, 144.33, 137.79, 127.80, 123.31, 122.78, 119.06, 103.29, 66.31, 57.96, 55.08, 43.04, 24.80. HRMS: m/z 515.31223 corresponds to molecular formula $C_{30}H_{38}N_6O_2H^+$ (error in ppm −1.30).

*N,N′-Bis[4-(morpholin-4-yl)buthyl]quinolino[8,7-h]-quinoline-1,7-diamine (**9**).* The general procedure provided above was followed using **6** (200.2 mg, 0.6692 mmol), (4-morpholin-4-ylbutyl)amine (870 mg, 5.50 mmol), and NMP (4 mL). The yield was 335.3 mg (92%). **9**: Light-brown powder, mp = 248−250 °C. IR (ATR): 3395m, 3088w, 2950m, 2861m, 2778w, 1596s, 1540s, 1473w, 1435w, 1342m, 1308w, 1246w, 1209w, 1147w, 1116m, 1072w, 1022w, 972w, 923w, 847m, 826w, 763w cm^{-1}. ^{1}H NMR (500 MHz, TFA): 8.87 (d, J = 9.4, 2H), 8.60 (d, J = 7.4, 2H), 8.57 (d, J = 9.4, 2H), 7.17 (d, J = 7.4, 2H), 4.41−4.37 (m, 4H), 4.15−4.09 (m, 4H), 3.90−3.85 (m, 4H), 3.82−3.78 (m, 4H), 3.49−3.38 (m, 8H), 2.21−2.06 (m, 8H). ^{13}C NMR (125 MHz, TFA): 159.11, 144.08, 137.82, 127.81, 123.25, 122.61, 118.95, 103.26, 66.37, 60.37, 55.06, 45.68, 26.90, 23.63. HRMS: m/z 543.34487 corresponds to molecular formula $C_{32}H_{42}N_6O_2H^+$ (error in ppm +1.23). Microanalysis for ($C_{32}H_{42}N_6O_2$): calculated: C 70.82; H 7.80; N 15.49; found: C 70.50; H 7.55; N 15.49.

*N,N′-Bis(2-thiomorpholin-4-ylethyl)quino[8,7-h]quinoline-1,7-diamine (**10**).* The general procedure provided above was followed using **6** (24.5 mg, 0.0819 mmol), (2-thiomorpholin-4-ylethyl)amine (120.0 mg, 0.8205 mmol), and NMP (1.3 mL). The yield was 39.2 mg (92%). **10**: Light-yellow powder, mp = 270−272 °C. IR (ATR): 3403m, 2913m, 2816m, 1594s, 1533s, 1459m, 1424m, 1330m, 1285w, 1241w, 1174w, 1123w, 1081w, 969w, 817w, 756m cm^{-1}. ^{1}H NMR (500 MHz, TFA): 8.94 (d, J = 9.4, 2H), 8.66 (d, J = 7.1, 2H), 8.56 (d, J = 9.4, 2H), 7.27 (d, J = 7.1, 2H), 4.37−4.31 (m, 4H), 4.16−4.10 (m, 4H), 3.87−3.81 (m, 4H), 3.49−3.41 (m, 4H), 3.27−3.18 (m, 4H), 2.90−2.83 (m, 4H). ^{13}C NMR (125 MHz, TFA): 159.10, 144.80, 137.79, 127.84, 123.37, 123.25, 119.30, 103.67, 58.06, 40.25, 26.75. HRMS: m/z 519.23577 corresponds to molecular formula $C_{28}H_{34}N_6S_2H^+$ (error in ppm −0.27).

*N,N′-Bis(3-thiomorpholin-4-ylpropyl)quino[8,7-h]-quinoline-1,7-diamine (**11**).* The general procedure provided above was followed using **6** (51.8 mg, 0.173 mmol), (3-thiomorpholin-4-ylpropyl)amine (277.6 mg, 1.732 mmol), and NMP (1.5 mL). The yield was 79.1 mg (84%). **11**: Light-yellow powder, mp = 262−264 °C. IR (ATR): 3274m, 2917m, 2816m, 1594s, 1539s, 1462m, 1428s, 1340m, 1283m, 1239m, 1172w, 1125m, 1086w, 1037w, 951w, 871w, 816m, 755m cm^{-1}. ^{1}H NMR (500 MHz, TFA): 8.88 (d, J = 9.4, 2H), 8.61 (d, J = 7.3, 2H), 8.57 (d, J = 9.4, 2H), 7.18 (d, J = 7.3, 2H), 4.06−4.00 (m, 4H), 3.94−3.87 (m, 4H), 3.59−3.52 (m, 4H), 3.41−3.33 (m, 4H), 3.27−3.18 (m, 4H), 2.91−2.83 (m, 4H), 2.58−2.49 (m, 4H). ^{13}C NMR (125 MHz, TFA): 159.03, 144.22, 137.67, 127.69, 123.21, 122.67, 118.95, 103.19, 58.21, 57.69, 43.02, 26.82, 24.78. HRMS: m/z 547.26705 corresponds to molecular formula $C_{30}H_{38}N_6S_2H^+$ (error in ppm −0.30).

*N,N′-Bis(4-thiomorpholin-4-ylbutyl)quino[8,7-h]quinoline-1,7-diamine (**12**).* The general procedure provided above was followed using **6** (83.2 mg, 0.278 mmol), (4-thiomorpholin-4-ylbutyl)amine (360 mg, 2.07 mmol), and NMP (2 mL). The yield was 158.3 mg (99%). **12**: Light-brown powder, mp = 226−228 °C. IR (ATR): 3415m, 3078w, 3018w, 2939m, 2864m, 2806m, 1594s, 1535s, 1472m, 1458m, 1425m, 1342s, 1283w, 1239m, 1206w, 1179w, 1108w, 1025w, 999w, 947w, 867w, 848w, 822m, 756m cm^{-1}. ^{1}H NMR (500 MHz, TFA): 8.89 (d, J = 9.4, 2H), 8.62 (d, J = 7.1, 2H), 8.60 (d, J = 9.4, 2H), 7.19 (d, J = 7.3, 2H), 4.07−4.01 (m, 4H), 3.92−2.86 (m, 4H), 3.46−3.35 (m, 8H), 3.31−3.22 (m, 4H), 2.95−2.88 (m, 4H), 2.22−2.06 (m, 8H). ^{13}C NMR (125 MHz, TFA): 159.08, 144.04, 137.78, 127.77, 123.21, 122.58, 118.91, 103.22, 60.71, 57.71, 45.65, 26.95, 23.67. HRMS: m/z 575.29796 corresponds to molecular formula $C_{32}H_{42}N_6S_2H^+$ (error in ppm −0.96).

*N,N′-Bis[2-(1,1-dioxidothiomorpholin-4-yl)ethyl]quino-[8,7-h]quinoline-1,7-diamine (**13**).* The general procedure provided above was followed using **6** (17.8 mg, 0.0595 mmol), 2-(1,1-dioxidothiomorpholin-4-yl)ethanamine (106.6 mg, 0.5980 mmol), and NMP (1.2 mL). The yield was 48.0 mg (98%). **13**: Light-brown powder, mp >280 °C. IR (ATR): 3408m, 2899w, 2828w, 1665w, 1594s, 1541m, 1465w, 1429w, 1389w, 1336m, 1293m, 1193w, 1127s, 1046w, 989w, 954w, 861w, 821w, 759w cm^{-1}. ^{1}H NMR (500 MHz, TFA): 9.00 (d, J

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ACS Infect. Dis. 2015, 1, 264−271

= 9.4, 2H), 8.71 (d, J = 7, 2H), 8.63 (d, J = 9.2, 2H), 7.34 (d, J = 7.2, 2H), 4.48−4.39 (m, 4H), 4.39−4.20 (m, 8H), 4.17−4.08 (m, 4H), 4.05−3.67 (m, 8H). ^{13}C NMR (125 MHz, TFA): 159.13, 144.85, 137.83, 127.88, 123.41, 123.35, 119.38, 103.78, 57.63, 54.61, 50.22, 40.81. HRMS: m/z 583.21510 corresponds to molecular formula $C_{28}H_{34}N_6O_4S_2H^+$ (error in ppm −0.81).

*N,N′-Bis[3-(1,1-dioxidothiomorpholin-4-yl)propyl]quino-[8,7-h]quinoline-1,7-diamine (**14**).* The general procedure provided above was followed using **6** (20.3 mg, 0.0679 mmol), 3-(1,1-dioxidothiomorpholin-4-yl)propan-1-amine (130.3 mg, 0.6777 mmol), and NMP (1.5 mL). The yield was 40.6 mg (98%). **14**: Light-brown powder, mp >280 °C. IR (ATR): 3641w, 3590w, 3393m, 3093w, 2942w, 2830w, 1596s, 1539s, 1474w, 1432w, 1390w, 1347s, 1290m, 1241w, 1190w, 1130s, 1047w, 991w, 946w, 852w, 800w, 765w, 716w, 671w, 554w cm^{-1}. ^{1}H NMR (500 MHz, TFA): 9.05 (d, J = 9.4, 2H), 8.77 (d, J = 7.1, 2H), 8.75 (d, J = 9.4, 2H), 7.35 (d, J = 7.4, 2H), 4.52−4.43 (m, 4H), 4.25−4.13 (m, 8H), 4.12−4.05 (m, 4H), 3.98−3.89 (m, 4H), 3.83−3.74 (m, 4H), 2.80−2.69 (m, 4H). ^{13}C NMR (125 MHz, TFA): 159.10, 144.30, 137.75, 127.77, 123.27, 122.77, 119.04, 103.23, 57.67, 54.19, 50.34, 42.86, 25.39. HRMS: m/z 611.24510 corresponds to molecular formula $C_{30}H_{38}N_6O_4S_2H^+$ (error in ppm −2.90).

*1,1′-[Quino[8,7-h]quinoline-1,7-diyldi(imino)]bis(3-morpholin-4-ylpropan-2-ol) (**15**).* The general procedure provided above was followed using **6** (36.1 mg, 0.121 mmol), 1-amino-3-morpholin-4-ylpropan-2-ol (193.1 mg, 1.205 mmol), and NMP (1.5 mL). The yield was 55.0 mg (83%). **15**: Light-yellow powder, mp = 258−260 °C. IR (ATR): 3427s, 2924m, 2862m, 2821m, 1650w, 1594s, 1534s, 1457m, 1430w, 1340w, 1304w, 1239w, 1113s, 1069w, 1006w, 915w, 861w. 803w, 754w, 634w, 542w cm^{-1}. ^{1}H NMR (500 MHz, TFA): 8.85 (d, J = 9.4, 2H), 8.57 (d, J = 7.4, 2H), 8.56 (d, J = 9.4, 2H), 7.39 (d, J = 7.4, 2H), 4.93−4.87 (m, 2H), 4.38−4.31 (m, 2H), 4.21−4.10 (m, 4H), 4.06−4.01 (m, 2H), 3.95−3.85 (m, 4H), 3.83−3.78 (m, 2H), 3.70−3.62 (m, 4H), 3.58−3.50 (m, 2H), 3.49−3.41 (m, 2H). ^{13}C NMR (125 MHz, TFA): 157.13, 141.77, 135.26, 125.24, 120.81, 120.26, 116.61, 101.34, 63.50, 59.54, 54.18, 51.46, 46.46. HRMS: m/z 547.30247 corresponds to molecular formula $C_{30}H_{38}N_6O_4H^+$ (error in ppm −0.48). Microanalysis for ($C_{30}H_{38}N_6O_4 \times (1/3)H_2O$): calculated: C 65.20; H 7.05; N 15.21; found: C 65.28; H 6.79; N 15.21.

*N,N′-Bis[2-(morpholin-4-yl)ethyl]quinolino[8,7-h]-quinoline-1,7-diamine tetrahydrochloride (**5**) (**ZS48**).* The general procedure provided above was followed using **7** (10.0 mg, 0.021 mmol) and 40% HCl in dry MeOH (1 mL). The yield was 12.7 mg (98%). **5**: Light-brown powder, mp >290 °C. IR (ATR): 3383m, 3231m, 3130m, 3047m, 2974m, 2869m, 2671m, 2588m, 2468w, 1627s, 1578s, 1498m, 1449m, 1363w, 1334w, 1270w, 1228m, 1198w, 1131m, 1093m, 1032w, 1006w, 976w, 912w, 867w, 823m, 743m cm^{-1}. ^{1}H NMR (500 MHz, D_2O): 8.41 (d, J = 6.4, H−C(3) and H−C(9)), 8.09 (d, J = 9.0, 2H), 8.00 (d, J = 8.7, 2H), 7.14 (d, J = 6.5, 2H), 4.19 (bs, 4H), 4.10 (bs, 8H), 3.76 (bs, 4H), 3.62 (bs, 8H). ^{13}C NMR (125 MHz, D_2O): 155.36, 142.11, 134.09, 123.68, 120.94, 119.36, 115.61, 101.36, 63.70, 54.04, 52.22, 37.57. HRMS: m/z 487.28080 corresponds to molecular formula $C_{28}H_{34}N_6O_2H^+$ (error in ppm −1.65). HPLC purity: method A: RT 2.084, area 96.93%; method B: RT 2.069, area 96.66%.

*N,N′-Bis[3-(morpholin-4-yl)propyl]quinolino[8,7-h]-quinoline-1,7-diamine tetrahydrochloride (**16**).* The general procedure provided above was followed using **8** (10.0 mg, 0.021 mmol) and 40% HCl in dry MeOH (1 mL). The yield was 12.6 mg (98%). **16**: Light-yellow powder, mp >280 °C. IR (ATR): 3383s, 3262s, 2948m, 2717m, 2483w, 1625s, 1574m, 1498w, 1442m, 1343w, 1264w, 1226w, 1173w, 1125w, 1099w, 1050w, 949w, 875w, 826w, 743w, 646w cm^{-1}. ^{1}H NMR (500 MHz, D_2O): 8.43 (d, J = 7.1, 2H), 8.19 (ABq, J_{AB} = 9.3, 4H), 7.12 (d, J = 7.4, 2H), 4.20−4.15 (m, 4H), 3.92−3.84 (m, 4H), 3.84−3.79 (m, 4H), 3.67−3.62 (m, 4H), 3.49−3.43 (m, 4H), 3.33−3.26 (m, 4H), 2.40−2.32 (m, 4H). ^{13}C NMR (125 MHz, D_2O): 155.48, 141.55, 134.21, 123.94, 120.68, 119.57, 115.42, 100.81, 63.69, 54.42, 51.78, 40.51, 22.07. HRMS: m/z 515.31252 corresponds to molecular formula $C_{30}H_{38}N_6O_2H^+$ (error in ppm −0.73). HPLC purity: method C: RT 3.518, area 96.97%; method D: RT 2.193, area 98.82%.

*N,N′-Bis[4-(morpholin-4-yl)buthyl]quinolino[8,7-h]-quinoline-1,7-diamine tetrahydrochloride (**17**) (**ZS64**).* The general procedure provided above was followed using **9** (10.0 mg, 0.018 mmol) and 40% HCl in dry MeOH (1 mL). The yield was 12.6 mg (99%). **17**: Light-yellow powder, mp >280 °C. IR (ATR): 3372m, 2945s, 2610m, 2477w, 1624s, 1570s, 1502w, 1441m, 1359w, 1227w, 1105w, 969w, 905w, 870w, 824w, 747w cm^{-1}. ^{1}H NMR (500 MHz, D_2O): 8.28 (d, J = 7.1, 2H), 7.96 (ABq, J_{AB} = 9.2, 4H), 7.02 (d, J = 7.4, 2H), 4.22−4.18 (m 4H), 3.93−3.87 (m, 4H), 3.72−3.62 (m, 8H), 3.40−3.35 (m, 4H), 3.33−3.25 (m, 4H), 2.06−1.91 (m, 8H). ^{13}C NMR (125 MHz, D_2O): 156.75, 142.79, 135.33, 125.02, 122.14, 120.80, 116.55, 102.40, 65.35, 58.21, 53.28, 44.73, 26.12, 22.36. HRMS: m/z 543.34461 corresponds to molecular formula $C_{32}H_{42}N_6O_2H^+$ (error in ppm +0.76). HPLC purity: method C: RT 2.194, area 95.14%; method D: RT 2.165, area 98.81%.

*N,N′-Bis(2-thiomorpholin-4-ylethyl)quino[8,7-h]quinoline-1,7-diamine tetrahydrochloride (**18**).* The general procedure provided above was followed using **10** (10.0 mg, 0.019 mmol) and 40% HCl in dry MeOH (1 mL). The yield was 12.6 mg (98%). **18**: Light-yellow powder, mp >280 °C. IR (ATR): 3236m, 3131m, 2961m, 2701m, 2545m, 2364m, 1627s, 1577s, 1498m, 1476m, 1446m, 1368w, 1335m, 1296w, 1272w, 1226m, 1156w, 1098w, 1057w, 956w, 882w, 825m, 744w cm^{-1}. ^{1}H NMR (500 MHz, D_2O): 8.09 (bs, 2H), 7.65 (bs, 4H), 6.72 (bs, 2H), 3.87 (bs, 4H), 3.55 (bs, 8H), 3.46 (bs, 4H), 2.99 (bs, 8H). HRMS: m/z 519.23514 corresponds to molecular formula $C_{28}H_{34}N_6S_2H^+$ (error in ppm −1.48). HPLC purity: method C: RT 2.262, area 98.07%; method D: RT 2.192, area 97.41%.

*N,N′-Bis(3-thiomorpholin-4-ylpropyl)quino[8,7-h]-quinoline-1,7-diamine tetrahydrochloride (**19**).* The general procedure provided above was followed using **11** (10.0 mg, 0.018 mmol) and 40% HCl in dry MeOH (1 mL). The yield was 12.5 mg (98%). **19**: Light-yellow powder, mp >280 °C. IR (ATR): 3260s, 2950s, 2678s, 1625s, 1574s, 1497m, 1441s, 1341m, 1269w, 1224m, 1157w, 1107w, 921w, 826m, 743w cm^{-1}. ^{1}H NMR (500 MHz, D_2O): 8.41 (d, J = 6.8, 2H), 8.14 (ABq, J_{AB} = 9.2, 4H), 7.10 (d, J = 7.1, 2H), 3.98−3.87 (m, 4H), 3.82−3.77 (m, 4H), 3.47−3.41 (m, 4H), 3.40−3.30 (m, 4H), 3.21−3.11 (m, 4H), 3.00−2.89 (m, 4H), 2.40−2.29 (m, 4H). ^{13}C NMR (125 MHz, D_2O): 157.06, 143.16, 135.78, 125.51, 122.27, 121.14, 117.01, 102.43, 56.44, 55.52, 42.22, 26.06, 23.71. HRMS: m/z 547.26672 corresponds to molecular formula $C_{30}H_{38}N_6S_2H^+$ (error in ppm −0.90). HPLC purity: method C: RT 3.345, area 96.59%; method D: RT 2.199, area 98.24%.

*N,N′-Bis(4-thiomorpholin-4-ylbutyl)quino[8,7-h]quinoline-1,7-diamine tetrahydrochloride (**20**) (**ZS67**).* The general procedure provided above was followed using **12** (10.0 mg,

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0.017 mmol) and 40% HCl in dry MeOH (1 mL). The yield was 12.4 mg (99%). **20**: Light-yellow powder, mp >280 °C. IR (ATR): 3410s, 2952s, 2680m, 2577m, 1625s, 1575m, 1502w, 1442w, 1357w, 1290w, 1270w, 1228w, 923w, 823w, 747w, 576w cm^{-1}. ^1H NMR (500 MHz, D_2O): 8.26 (d, J = 7, 2H), 7.93 (ABq, J_{AB} = 9.2, 4H), 7.00 (d, J = 7.1, 2H), 3.94−3.88 (m, 4H), 3.70−3.64 (m, 4H), 3.38−3.31 (m, 8H), 3.30−3.12 (m, 4H), 2.99−2.92 (m, 4H), 2.08−1.89 (m, 8H). ^{13}C NMR (125 MHz, D_2O): 156.72, 142.79, 135.32, 125.00, 122.12, 120.78, 116.53, 102.39, 58.56, 55.37, 44.73, 26.20, 26.04, 22.38. HRMS: m/z 575.29851 corresponds to molecular formula $C_{32}H_{42}N_6S_2H^+$ (error in ppm −1.61). HPLC purity: method C: RT 3.265, area 96.04%; method D: RT 2.195, area 97.80%.

*N,N′-Bis[2-(1,1-dioxidothiomorpholin-4-yl)ethyl]quino-[8,7-h]quinoline-1,7-diamine tetrahydrochloride (**21**).* The general procedure provided above was followed using **13** (10.0 mg, 0.017 mmol) and 40% HCl in dry MeOH (1 mL). The yield was 12.2 mg (98%). **21**: Light-yellow powder, mp >280 °C. IR (ATR): 3230m, 2985m, 1620s, 1570m, 1501w, 1441w, 1347w, 1318m, 1281w, 1227w, 1134m, 976w, 945w, 736w cm^{-1}. ^1H NMR (500 MHz, D_2O): 8.77 (d, J = 9.3, 2H), 8.65 (d, J = 6.8, 2H), 8.52 (d, J = 9.3, 2H), 7.23 (d, J = 7, 2H), 4.19−4.16 (m, 4H), 3.96−3.90 (m, 8H), 3.73−3.66 (m, 12H). ^{13}C NMR (125 MHz, D_2O): 156.19, 142.05, 135.34, 125.15, 121.04, 120.40, 116.54, 101.03, 53.51, 51.01, 48.01, 38.51. HRMS: m/z 583.21541 corresponds to molecular formula $C_{28}H_{34}N_6O_4S_2H^+$ (error in ppm −0.29). HPLC purity: method C: RT 2.525, area 95.18%; method D: RT 2.173, area 99.05%.

*N,N′-Bis[3-(1,1-dioxidothiomorpholin-4-yl)propyl]quino-[8,7-h]quinoline-1,7-diamine Tetrahydrochloride (**22**).* The general procedure provided above was followed using **14** (10.0 mg, 0.016 mmol) and 40% HCl in dry MeOH (1 mL). The yield was 12.2 mg (98%). **22**: Light-yellow powder, mp >280 °C. IR (ATR): 3327m, 2965m, 1626s, 1571m, 1501w, 1471w, 1442w, 1401w, 1347w, 1308m, 1275w, 1228w, 1132m, 1095w, 824w, 744w cm^{-1}. ^1H NMR (500 MHz, D_2O): 8.47 (d, J = 7, 2H), 8.34 (d, J = 9.3, 2H), 8.24 (d, J = 9.3, 2H), 7.14 (d, J = 7.2, 2H), 4.03−3.97 (m, 8H), 3.87−3.81 (m, 4H), 3.76−3.71 (m, 8H), 3.60−3.54 (m, 4H), 2.42−2.33 (m, 4H). ^{13}C NMR (125 MHz, D_2O): 155.70, 141.64, 134.50, 124.26, 120.78, 119.77, 115.68, 100.86, 54.06, 50.91, 47.69, 40.45, 22.83. HRMS: m/z 611.24668 corresponds to molecular formula $C_{30}H_{38}N_6O_4S_2H^+$ (error in ppm −0.31). HPLC purity: method C: RT 2.270, area 95.95%; method D: RT 2.238, area 95.18%.

*1,1′-[Quino[8,7-h]quinoline-1,7-diyldi(imino)]bis(3-morpholin-4-ylpropan-2-ol) Tetrahydrochloride (**23**) (**ZS62**).* The general procedure provided above was followed using **15** (10.0 mg, 0.018 mmol) and 40% HCl in dry MeOH (1 mL). The yield was 12.5 mg (98%). **23**: Light-yellow powder, mp >280 °C. IR (ATR): 3233s, 3128m, 3020m, 2968m, 2836s, 1623s, 1580s, 1500m, 1444m, 1374w, 1346w, 1314w, 1271w, 1228w, 1175w, 1109m, 1048w, 972w, 870w, 821w, 800w, 747w, 650w cm^{-1}. ^1H NMR (500 MHz, D_2O): 8.44 (d, J = 7.1, 2H), 8.22 (ABq, J_{AB} = 9.4, 4H), 7.21 (d, J = 7.4, 2H), 4.66−4.61 (m, 2H), 4.25−4.10 (m, 4H), 4.00−3.80 (m, 8H), 3.75−3.65 (m, 4H), 3.62−3.57 (m, 2H), 3.54−3.30 (m, 6H). ^{13}C NMR (125 MHz, D_2O): 157.77, 143.23, 135.93, 125.64, 122.34, 121.33, 117.12, 102.95, 65.02, 60.77, 54.98, 52.46, 48.45. HRMS: m/z 547.30249 corresponds to molecular formula $C_{30}H_{38}N_6O_4H^+$ (error in ppm −0.43). HPLC purity: method C: RT 3.445, area 97.64%; method D: RT 2.204, area 98.39%.

■ ANTIVIRAL ACTIVITY

In Vitro Studies. The anti-Ebola activity of the small molecules was determined using high-content imaging as described previously (refs 19 and 22) with some minor modifications. Briefly, HeLa cells (ATCC catalog no. CCL-2; 2000 cells/well) were seeded in 384-well Aurora optical imaging plates (Brooks/Nexus, catalog no. 1052-11130-S) and incubated overnight at 37 °C in 5% CO_2. The next day, cells were pretreated with appropriate concentrations of the compounds for 2 h and then infected with Ebola virus (Kikwit strain) at 0.5 multiplicity of infection (MOI). After 48 h postinfection, cells were fixed for 24 h with 10% formalin and then subjected to immunofluorescence staining. Infected cells were detected using viral-specific antiglycoprotein (GP) monoclonal antibody and Alexa 488 conjugated antimouse secondary antibody. The Hoechst dye was added to stain the nuclei, and Cellmask Deep red, to stain the cytoplasm. The cells were imaged used the Opera system (PerkinElmer), and image analysis was performed using Acapella software. The data was analyzed using Gene data software.

In Vivo Efficacy Studies. To test the in vivo efficacy of the compounds against EBOV, mice (BALB/c; n = 10/group) were treated with vehicle control or indicated concentrations of compounds **5** (**ZS48**), **17** (**ZS64**), **20** (**ZS67**), and **23** (**ZS62**) via the intraperitoneal (IP) route and after 2 h were infected via the IP route with 1000 pfu of the mouse-adapted strain of EBOV (Mayinga variant). Thereafter treatment was continued once daily for 6 days. Mice were monitored for survival for 14 days.

All of the tested compounds have a solubility of ≥25 mg/mL in deionized water. Research was conducted under an Institutional Animal Care and Use Committee approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.5b00028.

Synthesis and characterization of starting material, NMR spectra of all tested compounds, and two procedures for the determination of the purity of tested compounds (PDF)

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Notes

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The authors declare no competing financial interest.

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■ ABBREVIATIONS

EBOV, Ebola virus; ZEBOV, Ebola Zaire virus; MW, microwave reactor

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