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# Synthesis and pharmacological evaluation of choroquine derivatives bearing long aminated side chains as antivirus and anti-inflammatory agents

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ABSTRACT

Starting from the antimalarial drugs chloroquine and hydroxychloroquine, we conducted a structural optimization on the side chain of chloroquine by introducing amino substituted longer chains thus leading to a series of novel aminochloroquine derivatives. Anti-infectious effects against SARS-Cov2 spike glycoprotein as well as immunosuppressive and anti-inflammatory activities of the new compounds were evaluated. Distinguished immunosuppressive activities on the responses of T cell, B cell and macrophages upon mitogen and pathogenic signaling were manifested. Compounds **9**–**11** displayed the most promising inhibitory effects both on cellular proliferation and on the production of multiple pro-inflammatory cytokines, including IL-17, IFN-γ, IL-6, IL-1β and TNF-α, which might be insightful in the pursuit of treatment for immune disorders and inflammatory diseases.

## 1. Introduction

The global public health crisis caused by the continuous spreading of coronavirus disease 2019 (COVID-19) pandemic due to the infection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spawned an astonishing number of morbidity and mortality worldwide. Interventions both prophylactic and therapeutic are therefore emergently needed. An earlier drug repurposing approach has proposed the antiviral drug remdesivir and the antimalarial drug chloroquine or hydroxychloroquine as potential treatment of COVID-19, [1–2] subsequently igniting tremendous investigations on their pathogenic mechanism and antivirus efficacy against this unique virus of SARS-CoV-2 [3,4].

Chloroquine has been a historical antimalarial drug for many centuries and continues to be used in combating rheumatoid arthritis, [5] systemic lupus erythematosus, [6] and other inflammatory diseases [7,8]. In the past decade, the underlying mechanisms of chloroquine and its hydroxyl analogue hydroxychloroquine in inflammation and rheumatic diseases have been revealed preliminarily [9]. Both drugs were found to interfere with lysosomal activity and autophagy, disrupt membrane stability, modulate immune signaling pathways and transcriptional activities, thus leading to suppression of cytokine release and amelioration of corresponding rheumatic and inflammatory diseases [9]. Meanwhile, the mechanism of chloroquine and hydroxychloroquine as potential treatment of COVID-19 was also proposed recently [10]. On the one hand, both drugs were found to reduce the infection of SARS-CoV-2 virus by blocking its entry through altering the glycosylation of the angiotensin converting enzyme 2 (ACE2) receptor and decreasing the affinity of spike protein of SARS-CoV-2 virus; On the other hand, both drugs were found to exert anti-inflammatory effects, thus effectively decrease the high concentrations of pro-inflammatory cytokines (IL-6, and TNF-α), as observed in the plasma of critically ill COVID-19

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patients. Unfortunately, in spite of these promising preclinical results, clinical investigations of these two drugs as anti-COVID-19 treatment provided no solid, even contradictory results, thus suspending them only as experimental drugs [11–14]. As a matter of fact, recent studies showed that the antivirus effect of chloroquine and hydroxychloroquine is relatively modest and the potential adverse effect due to their unique pharmacokinetic properties (high and deep tissue distribution in many organs, $T_{1/2}$ up to 50 days) is an important concern [9,15]. Therefore, other than riskily and aggressively designing clinical paradigms to achieve optimal balance between efficacy and safety, we recently conducted a structural optimization on the side chain of hydroxychloroquine by changing the hydroxyethyl to the aminoethyl motif bearing various longer *N*-substituents, thus leading to a series of aminochloroquines. Herein, we report the synthesis and biological evaluation of these new derivatives.

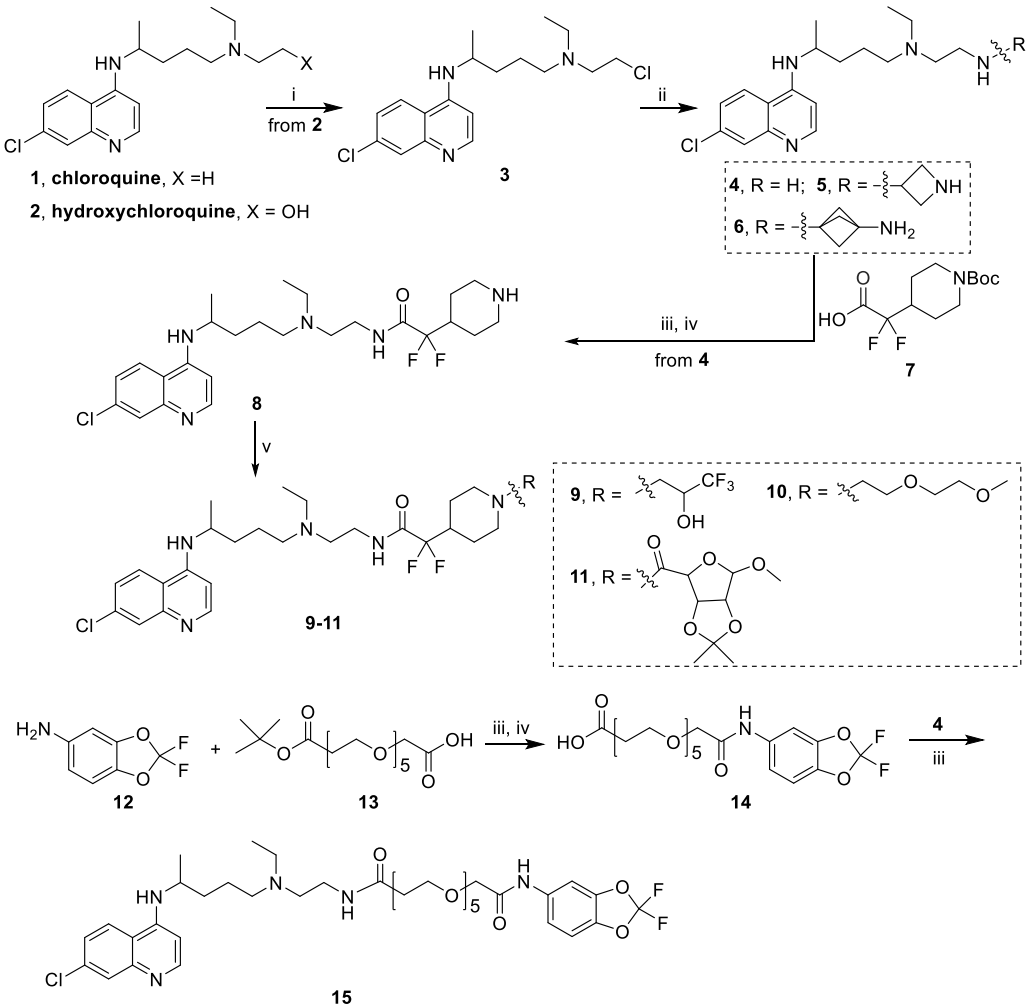
## 2. Results and discussion

### 2.1. Chemistry

The synthesis of aminated derivatives of chloroquine (**1**) bearing diversified substitutions was shown in Scheme 1. Treatment of hydroxychloroquine (**2**) with $SOCl_2$ afforded the intermediate **3** in quantitative yield. Substitution of chloride **3** with $NH_3{\cdot}H_2O$ or diverse *N*-protected amines followed by deprotection delivered aminated derivatives **4**–**6** in 58–65% yields. Condensation of the intermediate **4** with acid **7** using TBTU followed by deprotection afforded compound **8** in 72% yield. Substitution of **8** with diverse alkyl bromides in the presence of $K_2CO_3$ yielded amides **9**–**10** in 75% and 80% yields, respectively. In addition, condensation of **8** with 1-methoxy-2, 3-*O*-isopropylidene-*β*-D-ribofuranosyl-5-carboxylic acid afforded ribose substituted product **11** in 65% yield. The synthesis of compound **15** was facilitated in 34.5% overall yield by condensation of commercial available aniline **12** with acid **13** affording the intermediate acid **14**, which was then treated with amine **4**.

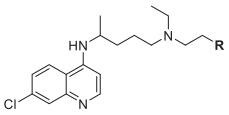
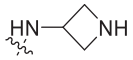
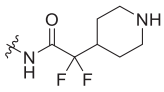
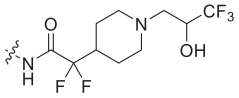
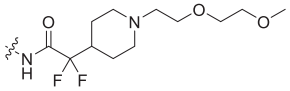
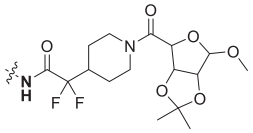
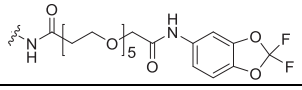
### 2.2. Anti-infectious effect of new compounds against SARS-CoV-2 spike glycoprotein

To quickly identify potent inhibitors of SARS-Cov2 spike glycoprotein, Huh7 cells infected with pseudo-virus particles (2019nCoV-S-HIV) packaged on 293 T cells were used to test the inhibitory activity of compounds against 2019nCoV-2 spike glycoprotein. As shown in Table 1, chloroquine (**1**) displayed a weak inhibitory effect on SARS-CoV-2 spike glycoprotein with an $EC_{50}$ value of 16.66 μM, which is only slightly lower than its cytotoxicity against Huh7 cell ($CC_{50}$: 26.36 μM). Nevertheless, hydroxychloroquine (**2**) was ineffective under this test condition. Although the biological data are different from the reported due to different test conditions, the overall outcomes are similar, confirming both chloroquine and hydroxychloroquine are weak




**Scheme 1.** [a]*Reaction and conditions*. (i) $SOCl_2$, DCM, rt; (ii) For **4**: 7 N $NH_3$ in MeOH, 80 °C; For **5** and **6**: a). *tert*-butyl 3-aminoazetidine-1-carboxylate or *tert*-butyl (3-aminobicyclo[1.1.1]pentan-1-yl)carbamate, DIPEA, EtOH, 80 °C; b) TFA, DCM; (iii) TBTU, DIPEA, DMF; (iv) TFA, DCM; (v) For **9** and **10**: $K_2CO_3$, DMF; For **11**: TBTU, DIPEA, DMF.

**Table 1**
Cytotoxicity and anti-infectious effects of compounds against SARS-Cov2 spike glycoprotein *

|  | R= | Huh7 cell | 2019nCoV-S-HIV |
|---|---|---|---|
| | | $CC_{50}$, μM | $EC_{50}$, μM |
| **1**, chloroquine | H | 26.36 | 16.66 |
| **2**, hydroxychloroquine | OH | > 80 | > 80 |
| **5** |  | > 80 | > 80 |
| **6** |  | 24.93 | 40.46 |
| **8** |  | 27.29 | 31.59 |
| **9** |  | 16.68 | 32.75 |
| **10** |  | 5.75 | 5.53 |
| **11** |  | 16.90 | 25.23 |
| **15** |  | 21.66 | 20.36 |

* $CC_{50}$ means the cytotoxic concentration of the compound that reduces cell viability by 50%; $EC_{50}$ means the concentration for 50% of maximal effect.

inhibitors of SARS-Cov2 spike glycoprotein. Compound **5** bearing an azetidin-3-amino and compound **6** bearing a bicyclo[1.1.1]pentane-1,3-diamino moieties as substituents of the hydroxyl in the side chain of hydroxychloroquine showed ineffective or much reduced potency with $EC_{50}$ values of > 80 μM and 40.46 μM, respectively. Compounds **8**–**11** represent a subseries of derivatives bearing a piperidinylsubstituted difluoro acylamino moiety. Again, all these compounds exhibited poor inhibitory activity with $EC_{50}$ values>20 μM, with an exception of compound **10** showing an improved inhibitory effect against 2019nCoV-2 spike glycoprotein with an $EC_{50}$ value of 5.53 μM. Unfortunately, the cytotoxicity of this compound was also increased by showing a similar $CC_{50}$ value (5.75 μM). Compound **15** bearing a PEGylated acylamino moiety to replace the hydroxyl in the side chain of hydroxychloroquine also showed marginal potency against the virus.

### 2.3. Immunosuppressive activity of new compounds

Since the new compounds did not show improved inhibitory effects against SARS-Cov2 spike glycoprotein, we turn to test their immunosuppressive activity on concanavalin A (Con A)-induced T cell proliferation and lipopolysaccharide (LPS)-induced B cell proliferation. As shown in Table 2, both chloroquine and hydroxychloroquine displayed moderate inhibitory effects against both T cell ($IC_{50}$ = 15.27 μM vs 18.20 μM) and B cell ($IC_{50}$ = 14.59 μM vs 14.16 μM) proliferation, in spite of their relative low cytotoxicity. The small heterocyclic amino substituted compound **5** lost activity completely and the alkylamino substituted compound **6** did not show better activity than chloroquine or hydroxychloroquine as well. Intriguingly, the acylamino substituted compounds **8**–**11** and **15** showed sharp difference in the anti-proliferative activity. Contrasting to the others, compound **8** bearing a 2,2-difluoro-2-(piperidin-4-yl)acetamino moiety displayed significantly compromised inhibitory activity against T cell and B cell proliferation with $IC_{50}$ values of 45.41 μM and 28.29 μM, respectively. However,

**Table 2**
Immunosuppressive activity of new compounds against Con A-and LPS-induced proliferation of murine splenocytes*

| Compound | Splenocytes Cytotoxicity | Con A-induced *T*-cell proliferation | | LPS-induced B-cell proliferation | |
|---|---|---|---|---|---|
| | $CC_{50}$, μM | $IC_{50}$, μM | SI# | $IC_{50}$, μM | SI |
| **1** | 25.54 ± 2.47 | 15.27 ± 0.13 | 1.67 | 14.59 ± 0.56 | 1.75 |
| **2** | 26.76 ± 2.81 | 18.20 ± 1.45 | 1.47 | 14.16 ± 0.21 | 1.89 |
| **5** | > 250 | > 250 | / | > 250 | / |
| **6** | 32.44 ± 6.22 | 16.71 ± 0.34 | 1.94 | 15.23 ± 0.46 | 2.12 |
| **8** | 71.63 ± 3.47 | 45.41 ± 1.43 | 1.58 | 28.29 ± 6.13 | 2.60 |
| **9** | 5.71 ± 1.04 | 2.85 ± 0.49 | 2.02 | 1.07 ± 0.23 | 5.66 |
| **10** | 8.10 ± 0.77 | 4.27 ± 0.33 | 1.91 | 1.42 ± 0.15 | 5.71 |
| **11** | 11.24 ± 0.08 | 2.49 ± 0.11 | 4.51 | 2.99 ± 0.73 | 3.90 |
| **15** | 6.07 ± 0.40 | 1.32 ± 0.06 | 4.58 | 1.44 ± 0.56 | 4.62 |

* $CC_{50}$ means the cytotoxic concentration of the compound that reduces cell viability by 50%; $IC_{50}$ means the inhibitory concentration of the compound that reduces cell proliferation by 50%; Data were the average values of three independent experiments.

# SI: selective index = $CC_{50}/IC_{50}$, determining whether the sample has biologically significant activity, SI>2 is considered to have biological effects.

compounds **9**–**11** bearing substitution on the terminal piperidine nitrogen with alkyl or acyl exhibited much improved inhibitory activities on both ConA-induced T cell proliferation and LPS-induced B-cells proliferation, with $IC_{50}$ values ranging from 2.49 – 4.27 μM and 1.07–2.99 μM, respectively. Interestingly, improvement in the immunosuppressive effect was also observed for compound **15** bearing a PEGylated acylamino moiety, with $IC_{50}$ values of 1.32 and 1.44 μM on T and B cells proliferation, respectively. Overall, compared with chloroquine and hydroxychloroquine, compounds **9**–**11** and **15** exhibited

significantly improved immunosuppressive activities.

### 2.4. Inhibitory effects of compounds on the production of t and B cell-derived cytokines

Since compounds **9**–**11** and **15** exhibited remarkable suppression against Con A-induced T cell proliferation and LPS-induced B cell proliferation, further investigation of their impacts on T and B cell-derived cytokines, the critical contributors in immune responses, was carried out in the splenocytes cultures stimulated by Con A (Table 3, Fig. 1A) and by LPS (Table 4, Fig. 1B). Although all of the four new compounds as well as chloroquine and hydroxychloroquine suppressed cytokine production from Con A- and LPS-stimulated splenocytes to a similar extent when estimated by $IC_{50}$ values, the new compounds (**9**–**11** and **15**) showed superior inhibition on LPS-induced IL-6 and IL-1β production from splenocytes. Particularly, compound **11** displayed distinguished suppressive activities even in a lower range of concentration. Concentration-dependent inhibition by this compound was observed from 3.90 μM down on the production of inflammatory cytokines including IL-17, IFN-γ and IL-6 from Con A- (Fig. 1A) and IL-1β from LPS-stimulated (Fig. 1B) splenocytes without overt cytotoxicity (cell survival rate over 95%), the inhibitory rates achieved by chloroquine and hydroxychloroquine at the same concentrations were only half of the rates by compound **11**.

### 2.5. Inhibitory effects of compounds on the production of macrophage-derived cytokines

Macrophages are major source of inflammatory cytokines involved in immune response, inflammation and infectious processes. Upon triggered by micro-organisms, microbial products or endogenous factors, macrophages can de novo synthesize and release various cytokines. We therefore examined the impact of compounds on LPS-induced cytokine production from the murine macrophages RAW 264.7 cells. As shown in Table 5, compound **11** performed much more potent restriction on the production of IL-6, IL-1β and TNF-α ($IC_{50}$ = 1.87 μM, 1.19 μM, and4.41 μM) than **1** ($IC_{50}$ = 14.63 μM, 2.72 μM, and 35.02 μM) and **2** ($IC_{50}$ = 16.54 μM, 3.51 μM, and 10.03 μM), whereas compounds **9** and **10** exerted better inhibition on IL-6 and IL-1β.

## 3. Conclusions

In summary, the antimalarial drugs chloroquine and hydroxychloroquine have been claimed as potential treatment of COVID-19 but lack solid and systemic clinical outcomes. In view of the fact that chloroquine and its *N*-ethyl side chain hydroxylated analogue hydroxychloroquine have similar potency as anti-inflammatory drugs but the latter showing better pharmacokinetic properties and improved selective index, in this report we set out to synthesize a small series of compounds by replacing the side chain hydroxyl group in hydroxychloroquine with diversely functionalized alkylamino or acylamino motifs. The new aminated derivatives (aminochloroquines) manifested distinguished suppression on T and B cell proliferation and production of multiple pro-inflammatory cytokines, including IL-17, IFN-γ, IL-6, IL-1β and TNF-α, derived from T cells, B cells and macrophages, upon mitogen or pathogenic signaling activation. Although the pharmacokinetic properties may not be improved due to the incorporation of the amido moiety and the longer chain, our findings offer a prospect for developing compounds **9**–**11** as lead compounds for the search of treatment for immune disorders and inflammatory diseases.

**Table 3**
Inhibitory effects of compounds on Con A-induced cytokine production.

| Comp. | $IC_{50}$*, μM | | |
|---|---|---|---|
| | IFN-γ | IL-17 | IL-6 |
| **1** | 0.65 ± 0.31 | 0.65 ± 0.28 | 7.20 ± 1.49 |
| **2** | 0.44 ± 0.01 | 0.62 ± 0.23 | 5.78 ± 2.39 |
| **9** | 1.23 ± 0.23 | 1.04 ± 0.20 | 0.16 ± 0.08 |
| **10** | 0.61 ± 0.34 | 0.25 ± 0.03 | 2.70 ± 1.16 |
| **11** | 0.35 ± 0.18 | 0.17 ± 0.06 | 0.45 ± 0.18 |
| **15** | 1.67 ± 0.44 | 1.76 ± 0.72 | 0.47 ± 0.16 |

* $IC_{50}$ means the inhibitory concentration of the compound that reduces cytokines secretion by 50%; Data were the average values of three independent experiments.

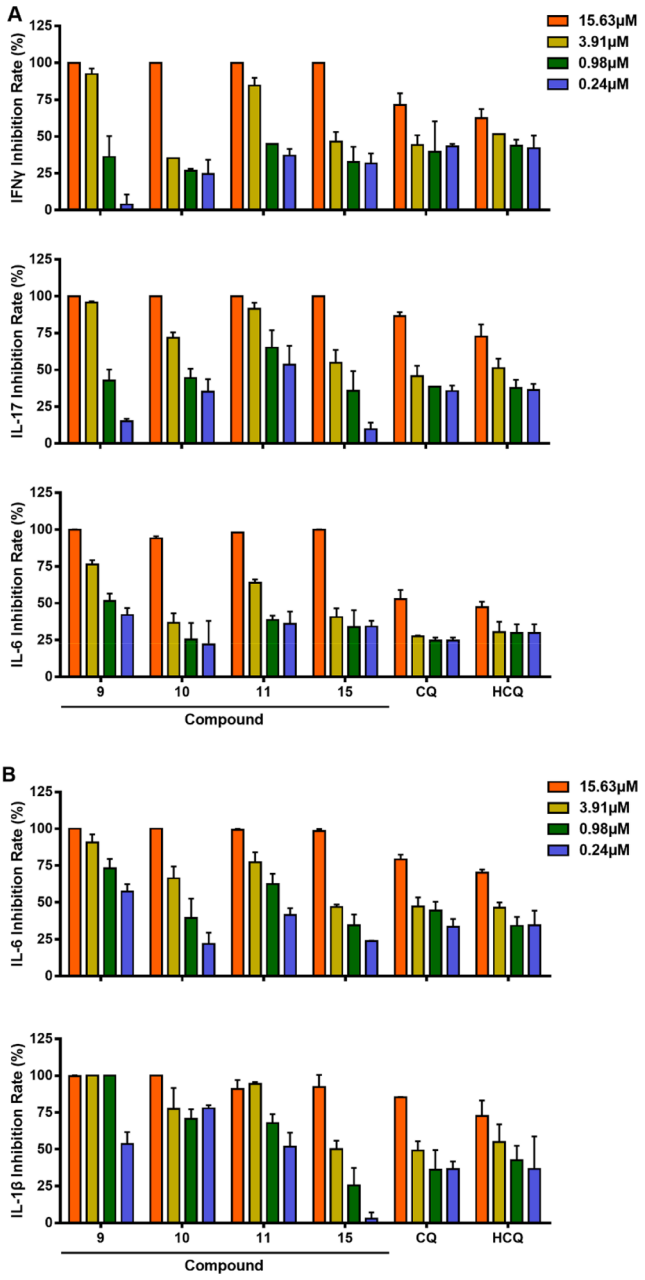



**Fig. 1. Inhibitory effects of compounds on cytokines production from Con A- or LPS-stimulated splenocytes.** Splenocytes were cultured with (A) Con A (1 μg/mL) or (B) LPS (10 μg/mL) in the absence or presence of compounds (15.63, 3.91, 0.98 and 0.24 μM) in 96-well plates for 48 h. The culture supernatants were collected and then quantified for IFN-γ, IL-17, IL-6 and IL-1β production by ELISA. Data were shown as mean ± SD. CQ: chloroquine; HCQ: hydroxychloroquine.

**Table 4**
Inhibitory effects of compounds on LPS-induced cytokine production.

| Comp. | $IC_{50}$*, μM | |
|---|---|---|
| | IL-6 | IL-1β |
| **1** | 0.81 ± 0.78 | 0.48 ± 0.34 |
| **2** | 0.86 ± 0.57 | 1.49 ± 0.32 |
| **9** | 0.01 ± 0.01 | 0.21 ± 0.08 |
| **10** | 1.48 ± 0.81 | 0.003 ± 0.002 |
| **11** | 0.17 ± 0.11 | 0.04 ± 0.03 |
| **15** | 1.51 ± 0.53 | 3.34 ± 0.62 |

* $IC_{50}$ means the inhibitory concentration of the compound that reduces cytokines secretion by 50%; Data were the average values of three independent experiments.

**Table 5**
Cytotoxicity and inhibitory effects on cytokine production in LPS-induced RAW264.7 cell*.

| Comp. | Cytotoxicity | IL-6 | | IL-1β | | TNF-α | |
|---|---|---|---|---|---|---|---|
| | $CC_{50}$, μM | $IC_{50}$, μM | SI | $IC_{50}$, μM | SI | $IC_{50}$, μM | SI |
| **1** | 21.95 ± 3.59 | 14.63 ± 1.27 | 1.42 | 2.72 ± 0.75 | 7.65 | 35.02 ± 18.73 | 0.81 |
| **2** | 25.34 ± 4.38 | 16.54 ± 0.55 | 1.63 | 3.51 ± 0.15 | 7.74 | 10.03 ± 2.97 | 2.69 |
| **9** | 48.64 ± 0.18 | 1.61 ± 0.25 | 30.62 | 0.72 ± 0.17 | 69.74 | 15.89 ± 0.45 | 3.06 |
| **10** | 125.20 ± 45.73 | 2.52 ± 0.36 | 50.18 | 1.81 ± 0.83 | 77.64 | 24.36 ± 6.05 | 5.56 |
| **11** | 47.71 ± 0.15 | 1.87 ± 0.44 | 26.39 | 1.19 ± 0.41 | 43.03 | 4.41 ± 1.66 | 12.27 |
| **15** | 44.7 6 ± 0.03 | 9.63 ± 3.75 | 5.10 | 3.84 ± 1.25 | 12.72 | 10.74 ± 3.99 | 4.52 |

* $CC_{50}$ means the cytotoxic concentration of the compound that reduces cell viability by 50%; $IC_{50}$ means the inhibitory concentration of the compound that reduces cytokines secretion by 50%; Data were the average values of three independent experiments.

## 4. Experimental section

### 4.1. Chemical reagents and general method

All commercially available starting materials and solvents are reagent grade and used without further purification. Column chromatography was performed using 300–400 mesh or 200–300 mesh silica gel. Analytical TLC was carried out employing silica gel 60 F254 plates and spots were visualized by UV (254 or 365 nm). $^1H$ and $^{13}C$ NMR spectra were recorded with a Varian-MERCURY 300 or 400 MHz NMR spectrometer. Chemical shifts (δ) were reported in ppm downfield from an internal TMS standard and *J* values were given in Hz. Low- and high-resolution mass spectra were obtained in the ESI mode from an Elite mass spectrometer.

### 4.2. Chemistry

#### 4.2.1. $N^1$-(2-Chloroethyl)-$N^4$-(7-chloroquinolin-4-yl)-$N^1$-ethylpentane-1,4-diamine (3)

To a solution of compound **2** (6.72 g, 20.0 mmol) in DCM (80 mL) at 0 °C was added $SOCl_2$ (20 mL) dropwise. The mixture was stirred at room temperature for 2 h and then concentrated in vacuo. Ethanol was added to the residue and the solution was concentrated in vacuo. After repeating the operation twice to remove the excess $SOCl_2$, the yellowish oil **3** was obtained and used in next step without further purification.

#### 4.2.2. $N^1$-(2-Aminoethyl)-$N^4$-(7-chloroquinolin-4-yl)-$N^1$-ethylpentane-1,4-diamine (4)

To a solution of compound **3** (3.54 g, 10.0 mmol) in EtOH (20 mL) was added 7 N $NH_3$ in MeOH (10 mL). The mixture was stirred at 80 °C in a sealed tube for 2 h and then concentrated in vacuo. Ethanol was added to the residue and the solution was concentrated. The crud product **4** was obtained as colorless oil and used in next step without further purification.

#### 4.2.3. General procedure for synthesis of compounds 5 and 6

The solution of compound **3** (353 mg, 1 mmol), DIPEA (258 mg, 2 mmol), *tert*-butyl 3-aminoazetidine-1-carboxylate (258 mg, 1.5 mmol) or *tert*-butyl (3-aminobicyclo[1.1.1]pentan-1-yl)carbamate (297 mg, 1.5 mmol) in EtOH (10 mL) was stirred at 80 °C for 1 h. The mixture was concentrated in vacuo and the residue was purified by silica gel column to yield the *N*-protected intermediates. The intermediate was disolved in DCM (10 mL) and $CF_3COOH$ (2 mL) was added. The reaction was stirred at room temperature for 1 h and then concentrated in vacuo. The residue was purified by silica gel column to afford the desired compound **5** or **6** in 58–65% yields (two steps).

#### 4.2.4. $N^1$-(2-(Azetidin-3-ylamino)ethyl)-$N^4$-(7-chloroquinolin-4-yl)-$N^1$-ethylpentane-1,4-diamine (5)

White bubbly solid (225 mg, 58% yield). $^1H$ NMR (400 MHz, $CD_3OD$) $\delta$ 8.33 (d, $J = 5.7$ Hz, 1H), 8.18 (d, $J = 9.0$ Hz, 1H), 7.76 (d, $J = 2.0$ Hz, 1H), 7.39 (dd, $J = 9.0$, 2.2 Hz, 1H), 6.56 (d, $J = 5.8$ Hz, 1H), 3.83 (d, $J = 5.7$ Hz, 1H), 3.64 – 3.49 (m, 3H), 3.35 (t, $J = 6.5$ Hz, 2H), 2.50 (m, 8H), 1.80 – 1.69 (m, 1H), 1.63 (m, 3H), 1.32 (d, $J = 6.4$ Hz, 3H), 1.00 (t, $J = 7.1$ Hz, 3H). $^{13}C$ NMR (126 MHz, $CD_3OD$) $\delta$ 152.50, 152.20, 149.97, 136.34, 127.64, 125.87, 124.46, 118.86, 99.88, 55.19, 54.61, 54.17, 53.80, 49.49, 48.76, 44.95, 35.09, 24.63, 20.45, 11.71. MS (ESI, $[M + H]^+$) *m/z* 390.3; HRMS (ESI) calcd for $C_{21}H_{33}ClN_5^+$ $[M + H]^+$ 390.2419; found, 390.2421.

#### 4.2.5. $N^1$-(2-((4-((7-Chloroquinolin-4-yl)amino)pentyl)(ethyl)amino)ethyl)bicyclo[1.1.1]pentane-1,3-diamine (6)

White bubbly solid (270 mg, 65% yield). $^1H$ NMR (400 MHz, $CD_3OD$) $\delta$ 8.34 (d, $J = 5.8$ Hz, 1H), 8.21 (d, $J = 9.0$ Hz, 1H), 7.76 (d, $J = 2.1$ Hz, 1H), 7.40 (dd, $J = 9.0$, 2.2 Hz, 1H), 6.57 (d, $J = 5.9$ Hz, 1H), 3.84 (s, 1H), 2.74 – 2.51 (m, 8H), 1.76 (s, 6H), 1.70 (m, 1H), 1.68 – 1.53 (m, 3H), 1.33 (d, $J = 6.4$ Hz, 3H), 1.04 (t, $J = 7.2$ Hz, 3H). $^{13}C$ NMR (126 MHz, $CD_3OD$) $\delta$ 151.00, 150.70, 148.09, 135.14, 125.89, 124.61, 123.19, 117.37, 98.49, 53.07, 52.64, 52.17, 48.73, 47.53, 47.09, 45.44, 42.33, 33.47, 22.87, 19.03, 9.93. MS (ESI, $[M + H]^+$) *m/z* 416.3;HRMS (ESI) calcd for $C_{23}H_{35}ClN_5^+$ $[M + H]^+$ 416.2576; found, 416.2572.

#### 4.2.6. N-(2-((4-((7-Chloroquinolin-4-yl)amino)pentyl)(ethyl)amino)ethyl)-2,2-difluoro-2-(piperidin-4-yl)acetamide (8)

White bubbly solid (71 mg, 72% yield). The solution of compound **4** (67 mg, 0.2 mmol), acid **7** (84 mg, 0.3 mmol), TBTU (128 mg, 0.4 mmol) and DIPEA (77 mg, 0.6 mmol) in DMF (1 mL) was stirred at room temperature for 2 h under $N_2$ atmosphere. The reaction solution was poured into an ice water mixture. After extraction with EtOAc three times, the combined organic phase was washed with brine, dried over $Na_2SO_4$ and concentrated in vacuo. The residue was purified by silica gel column to afford the *N*-Boc protected intermediate. The intermediate was dissolved the in DCM (4 mL) and $CF_3COOH$ (1 mL) was added. The reaction was stirred at room temperature for 1 h and then concentrated in vacuo. The residue was further purified by silica gel column to afford the desired compound **8** in 72% yield (two steps). $^1H$ NMR (400 MHz, $CD_3OD$) $\delta$ 8.33 (d, $J = 5.7$ Hz, 1H), 8.24 (d, $J = 9.0$ Hz, 1H), 7.77 (s, 1H), 7.41 (d, $J = 9.0$ Hz, 1H), 6.59 (d, $J = 5.8$ Hz, 1H), 3.85 (d, $J = 5.1$ Hz, 1H), 3.34 (m, 4H), 2.84 (t, $J = 12.7$ Hz, 2H), 2.69 – 2.50 (m, 6H), 2.47 – 2.35 (m, 1H), 1.88 (d, $J = 13.6$ Hz, 2H), 1.82 – 1.51 (m, 6H), 1.33 (d, $J =$

6.3 Hz, 3H), 1.02 (t, $J$ = 7.1 Hz, 3H). $^{13}$C NMR (126 MHz, $CD_3OD$) $\delta$ 163.99 (t, $J$ = 29.2 Hz), 151.30, 150.15, 147.48, 135.38, 125.39, 124.73, 123.36, 117.51 (t, $J$ = 253.6 Hz), 117.25, 98.50, 53.06, 47.44, 51.35, 48.34, 43.43, 38.39 (t, $J$ = 23.2 Hz), 36.51, 33.50, 23.28, 22.20, 18.98, 10.36. MS (ESI, $[M + H]^+$) *m/z* 496.3.

#### 4.2.7. General procedure for synthesis of compounds 9 and 10

To a solution of compound **8** (64.3 mg, 0.13 mmol) and 3-bromo-1,1,1-trifluoropropan-2-ol (50.2 mg, 0.26 mmol) or 1-bromo-2-(2-methoxyethoxy)ethane (47.6 mg, 0.26 mmol) in DMF (3 mL) at room temperature was added $K_2CO_3$ (35.9 mg, 0.26 mmol). The mixture was stirred at room temperature for 2 h and then poured into ice water. After extraction with EtOAc three times, the combined organic phase was washed with brine, dried over $Na_2SO_4$, filtered, and concentrated in vacuo. The residue was further purified by silica gel column to afford the desired compound **9** or **10** in 75% or 80% yield, respectively.

#### 4.2.8. N-(2-((4-((7-Chloroquinolin-4-yl)amino)pentyl)(ethyl)amino) ethyl)-2,2-difluoro-2-(1-(3,3,3-trifluoro-2-hydroxypropyl)piperidin-4-yl) acetamide (9)

White bubbly solid (59.3 mg, 75% yield). $^1$H NMR (400 MHz, $CD_3OD$) $\delta$ 8.33 (d, $J$ = 5.9 Hz, 1H), 8.22 (d, $J$ = 9.0 Hz, 1H), 7.77 (d, $J$ = 2.1 Hz, 1H), 7.41 (dd, $J$ = 9.0, 2.2 Hz, 1H), 6.59 (d, $J$ = 6.0 Hz, 1H), 4.17 – 4.04 (m, 1H), 3.85 (m, 1H), 3.34 (m, 2H), 3.07 – 2.93 (m, 2H), 2.57 (m, 8H), 2.14 – 1.92 (m, 3H), 1.84 – 1.42 (m, 8H), 1.33 (d, $J$ = 6.4 Hz, 3H), 1.01 (t, $J$ = 7.1 Hz, 3H). $^{19}$F NMR (471 MHz, $CD_3OD$) $\delta$ −80.47, −80.48, −116.03. $^{13}$C NMR (126 MHz, $CD_3OD$) $\delta$ 164.19 (t, $J$ = 29.3 Hz), 150.81, 149.72, 147.06, 134.92, 124.99, 124.75 (q, $J$ = 282.0 Hz), 124.28, 122.83, 117.75 (t, $J$ = 252.8 Hz), 116.80, 98.03, 67.05 (q, $J$ = 29.6 Hz), 56.98, 52.89, 52.56, 51.84, 50.94, 47.89, 47.42, 39.23 (t, $J$ = 22.8 Hz), 35.99, 33.06, 23.40, 22.95, 18.51, 9.94. MS (ESI, $[M + H]^+$) *m/z* 608.4, HRMS (ESI) calcd for $C_{28}H_{40}ClF_5N_5O_2^+$, 608.2785; found, 608.2793.

#### 4.2.9. N-(2-((4-((7-Chloroquinolin-4-yl)amino)pentyl)(ethyl)amino) ethyl)-2,2-difluoro-2-(1-(2-(2-methoxyethoxy)ethyl)piperidin-4-yl) acetamide (10)

White bubbly solid (62.2 mg, 80% yield). $^1$H NMR (400 MHz, $CD_3OD$) $\delta$ 8.34 (d, $J$ = 6.1 Hz, 1H), 8.28 (d, $J$ = 9.0 Hz, 1H), 7.78 (d, $J$ = 2.0 Hz, 1H), 7.46 (dd, $J$ = 9.1, 2.1 Hz, 1H), 6.66 (d, $J$ = 6.2 Hz, 1H), 3.96 – 3.84 (m, 1H), 3.63 (t, $J$ = 5.5 Hz, 2H), 3.58 (dd, $J$ = 6.0, 3.0 Hz, 2H), 3.53 (dd, $J$ = 6.0, 3.1 Hz, 2H), 3.35 (s, 3H), 3.15 (d, $J$ = 12.4 Hz, 2H), 2.70 (t, $J$ = 5.5 Hz, 2H), 2.62 (m, 6H), 2.23–2.12 (m, 3H), 1.85 – 1.49 (m, 8H), 1.35 (d, $J$ = 6.4 Hz, 3H), 1.04 (t, $J$ = 7.1 Hz, 3H). $^{13}$C NMR (126 MHz, $CD_3OD$) $\delta$ 164.46 (t, $J$ = 29.2 Hz), 152.09, 148.73, 145.87, 136.15, 125.20, 124.21, 123.63, 117.96 (t, $J$ = 253.0 Hz), 116.95, 98.54, 71.52, 69.78, 67.29, 57.71, 57.01, 52.98, 52.48, 51.32, 48.69, 47.53, 39.20 (t, $J$ = 23.0 Hz), 36.32, 33.37, 23.22, 18.92, 10.23. MS (ESI, $[M + H] +$) *m/z* 598.4, HRMS (ESI) calcd for $C_{30}H_{47}ClF_2N_5O_3^+$, 598.3330; found, 598.3332.

#### 4.2.10. N-(2-((4-((7-Chloroquinolin-4-yl)amino)pentyl)(ethyl)amino) ethyl)-2,2-difluoro-2-(1-(6-methoxy-2,2-dimethyltetrahydrofuro[3,4-d] [1,3]dioxole-4-carbonyl)piperidin-4-yl)acetamide (11)

White bubbly solid (58.8 mg, 65% yield). A solution of compound **8** (64.3 mg, 0.13 mmol), 1-methoxy-2,3-*O*-isopropylidene-$\beta$-D-ribofuranosyl-5-carboxylic acid (56 mg, 0.26 mmol), TBTU (83 mg, 0.26 mmol) and DIPEA (50 mg, 0.39 mmol) in DMF (3 mL) was stirred at room temperature for 4 h under $N_2$ atmosphere. The reaction solution was poured into ice water. After extraction with EtOAc three times, the combined organic phase was washed with brine, dried over $Na_2SO_4$, filtered, and concentrated in vacuo. The residue was further purified by silica gel column to afford the desired compound **11** in 65% yield. $^1$H NMR (400 MHz, $CD_3OD$) $\delta$ 8.34 (d, $J$ = 6.0 Hz, 1H), 8.25 (d, $J$ = 9.0 Hz, 1H), 7.77 (d, $J$ = 2.0 Hz, 1H), 7.44 (d, $J$ = 9.0 Hz, 1H), 6.63 (d, $J$ = 6.0 Hz, 1H), 5.37 (m, 1H), 4.92 (d, $J$ = 7.6 Hz, 1H), 4.83 (d, $J$ = 2.2 Hz, 1H), 4.57 (m, 1H), 4.48 (s, 1H), 4.29 (d, $J$ = 13.5 Hz, 0.5H), 4.20 (d, $J$ = 12.1 Hz, 0.5H), 3.88 (d, $J$ = 5.8 Hz, 1H), 3.34 (m, 1.6H), 3.22 (s, 1.3H), 3.12 (t, $J$ = 12.7 Hz, 1H), 3.00 (t, $J$ = 13.3 Hz, 1H), 2.68 – 2.48 (m, 6H), 2.48 – 2.28 (m, 1H), 1.88 – 1.51 (m, 6H), 1.43 (d, $J$ = 2.9 Hz, 3H), 1.34 (d, $J$ = 6.4 Hz, 3H), 1.31 (d, $J$ = 3.0 Hz, 3H), 1.02 (t, $J$ = 7.1 Hz, 3H). $^{13}$C NMR (126 MHz, $CD_3OD$) $\delta$ 168.80 and 168.65 (1C), 164.32 (t, $J$ = 28.5 Hz), 151.73, 149.36, 146.58, 135.83, 125.01, 124.75, 123.43, 117.88 (t, $J$ = 252.9 Hz), 117.08, 112.04, 110.05 and 109.37 (1C), 98.52, 85.20 and 84.98 (1C), 82.64 and 82.33 (1C), 81.21 and 81.13 (1C), 54.85 and 54.00 (1C), 53.05 and 53.00 (1C), 51.41, 48.54, 47.42, 44.84 and 44.53 (1C), 41.03 and 40.82 (1C), 39.97, 36.45, 33.47, 25.41 and 25.34 (1C), 24.56 and 224.36 (1C), 23.91 and 23.79 (1C), 23.72 and 23.67(1C), 23.37, 18.94, 10.32. MS (ESI, $[M + H] +$) *m/z* 696.4, HRMS (ESI) calcd for $C_{34}H_{49}$ Cl $F_2N_5O_6^+$, 696.3334; found, 696.3349.

#### 4.2.11. 1-((2,2-Difluorobenzo[d][1,3]dioxol-5-yl)amino)-1-oxo-3,6,9,12,15-pentaoxaoctadecan-18-oic acid **(14)**

A solution of compound **12** (35 mg, 0.2 mmol), acid **13** (76 mg, 0.2 mmol), TBTU (128 mg, 0.4 mmol) and DIPEA (77 mg, 0.6 mmol) in DMF (1 mL) was stirred at room temperature for 2 h under $N_2$ atmosphere. The reaction solution was poured into ice water. After extraction with EtOAc three times, the combined organic phase was washed with brine, dried over $Na_2SO_4$, filtered, and concentrated in vacuo. The residue was further purified by silica gel column to afford a *N*-protected intermediate. The intermediate was dissolved in DCM (4 mL) and $CF_3COOH$ (1 mL) was added. The reaction was stirred at room temperature for 1 h and then concentrated in vacuo. The crud product **14** was obtained in 51% yields (two steps) as light yellow oil which was used in next step without further purification.

#### 4.2.12. $N^{18}$-(2-((4-((7-Chloroquinolin-4-yl)amino)pentyl)(ethyl)amino) ethyl)-N1-(2,2-difluorobenzo[d][1,3]dioxol-5-yl)-3,6,9,12,15-pentaoxaoctadecanediamide (15)

Colorless oil (54 mg, 68%). A solution of compound **4** (34 mg, 0.1 mmol), acid **14** (48 mg, 0.1 mmol), TBTU (64 mg, 0.2 mmol) and DIPEA (39 mg, 0.3 mmol) in DMF (2 mL) was stirred at room temperature for 2 h under $N_2$ atmosphere. The reaction solution was then poured into ice water. After extraction with EtOAc three times, the combined organic phase was washed with brine, dried over $Na_2SO_4$, filtered, and concentrated in vacuo. The residue was further purified by silica gel column to afford the desired compound **15** in 68% yields (two steps). $^1$H NMR (400 MHz, $CD_3OD$) $\delta$ 8.34 (d, $J$ = 5.9 Hz, 1H), 8.22 (d, $J$ = 9.1 Hz, 1H), 7.77 (d, $J$ = 1.9 Hz, 1H), 7.69 (d, $J$ = 2.0 Hz, 1H), 7.43 (dd, $J$ = 9.1, 2.2 Hz, 1H), 7.30 (dd, $J$ = 8.7, 2.1 Hz, 1H), 7.14 (d, $J$ = 8.7 Hz, 1H), 6.61 (d, $J$ = 6.2 Hz, 1H), 4.12 (s, 2H), 3.86 (m, 1H), 3.77 – 3.49 (m, 18H), 3.28 (d, $J$ = 7.3 Hz, 2H), 2.65 (m, 6H), 2.37 (t, $J$ = 6.1 Hz, 2H), 1.83 – 1.55 (m, 4H), 1.33 (d, $J$ = 6.4 Hz, 3H), 1.05 (t, $J$ = 7.1 Hz, 3H). $^{13}$C NMR (151 MHz, $CD_3OD$) $\delta$ 172.38, 169.21, 150.93, 149.53, 146.79, 142.89, 139.47, 135.08, 133.77, 131.30 (t, $J$ = 253.0 Hz), 124.82, 124.40, 122.91, 116.75, 115.18, 108.70, 102.48, 98.08, 70.17, 69.69, 69.63, 69.59, 69.53, 69.46, 69.42, 69.40, 66.26, 52.51, 51.45, 47.88, 47.13, 35.77, 35.71, 32.91, 22.38, 18.51, 9.45. MS (ESI, $[M + H]^+$) *m/z* 796.5, HRMS (ESI) calcd for $C_{38}H_{53}ClF_2N_5O_9^+$, 796.3494; found, 796.3500.

### 4.3. Biochemistry

#### 4.3.1. Materials

Compounds were dissolved with 100% dimethylsulfoxide (Sigma-Aldrich, St. Louis, Missouri, USA) and then diluted with the RPMI 1640 medium (Gibco, NY, US) or Dulbecco's modified Eagle's medium (DMEM) (Gibco, NY, US) containing 10% fetal bovine serum (FBS, Hyclone). 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Concanavalin A (Con A) and LPS (055: B5) were purchased from Sigma (St Louis, MO, USA).

#### 4.3.2. Animals

Inbred 6 to 8-week-old female BALB/c mice were purchased from Shanghai Jiesijie Experimental Animal Co., Ltd. (Certificate No. 2018–0004, China). The mice were housed under specific pathogen-free conditions with a controlled environment (12 h of light/12 h of dark cycle, 22 ± 1 °C, 55 ± 5% relative humidity). All mice were fed standard laboratory chow and water ad libitum and allowed to acclimatize in our facility for one week before any experiments started. All experiments were carried out according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Bioethics Committee of the Shanghai Institute of *Materia Medica* (IACUC Protocol #2020-03-ZJP-120).

#### 4.3.3. Splenocytes preparation

Female BALB/c mice were sacrificed, and the spleens were removed aseptically. The spleens were ground up, and a single cell suspension was prepared after cell debris and clumps were removed. Erythrocytes were depleted with ammonium chloride buffer solution (0.155 M $NH_4Cl$ and 16.5 mM Tris, pH 7.2). Cells were washed and resuspended in RPMI 1640 media containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/ mL). Cells were counted by trypan blue exclusion.

#### 4.3.4. RAW264.7 cell cultures

Murine adherent macrophage cell line RAW264.7 cells were purchased form American Type Culture Collection (ATCC, Manassas, VA, USA). RAW264.7 cells were cultured in DMEM containing 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 ℃ humidified incubator containing 5% CO2.

#### 4.3.5. Cell viability

To evaluate the cytotoxicity of compounds, cell viability assays were performed using the MTT assay as previously reported [16]. Briefly, $8 \times 10^5$ (splenocytes) or $1 \times 10^5$ (RAW264.7) cells were cultured in triplicate for 48 h in the presence or absence of the compounds. MTT (5 mg/mL) reagent was added 4 h before the end of culture, and then the formazan was dissolved in DMSO. Absorbance was measured at 570 nm with a microplate reader (Spectramax 190, Molecular Devices Corporation, Sunnyvale, CA).

#### 4.3.6. ConA- and LPS-Induced proliferation assay

Splenocytes ($5 \times 10^5$ cells) were cultured in triplicate for 48 h, in the presence or absence of the compounds, and were stimulated with 1 μg/mL concanavalin A (ConA) to induce T cell proliferation and 10 μg/mL LPS to induce B cell proliferation. Cells were pulsed with 0.5 μCi/well of [$^3$H] thymidine for 8 h and harvested onto glass fiber filters. The incorporated radioactivity was counted using a Beta Scintillation Counter (MicroBeta TriLux, PerkinElmer Life Sciences, Boston, MA).

#### 4.3.7. Cytokines production assay

For splenocytes, $5 \times 10^5$ cells were seeded in 96-well plates in triplicates, in the presence or absence of the compounds, and were supplemented with ConA (1 μg/mL) or LPS (10 μg/mL) for 48 h. For RAW 264.7 cells, $1 \times 10^5$ cells were seeded in 96-well plates in triplicates, in the presence or absence of the compounds, and then were supplemented with LPS (10 μg/mL) for 48 h. Supernatants were collected for cytokines assays.

#### 4.3.8. ELISA

Cytokines in culture supernatants were determined by using mouse IFN-γ, IL-17, IL-6, TNF-α ELISA kits (BD Pharmingen) and IL-1β ELISA kit (Thermo Scientific, MA, US) according to the manufacturer's instructions.

#### 4.3.9. SARS-CoV2 spike protein entry activity assay

Huh-7 cells were obtained from The Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Vero E6 and 293 T cell lines were obtained from the ATCC (USA). All cell lines were grown in DMEM supplemented with 10% FBS.

To produce viral spike protein pseudotyped HIV virions, an HIV backbone vector pNL4-3. Luc.R-E- was used for pseudovirus packaging. 293 T cells were co-transfected with the respective envelop protein expression plasmid plus pNL4.3LucR-E- plasmid. At 72 h post-transfection, supernatants were harvested and stored at −80 °C.

For SARS2 spike protein pseudotyped virus entry assay, Huh-7 cells were pre-seeded in 96 well white and transparent plates respectively and incubated overnight. On next day, 50 μL medium containing desired concentration of compounds were transferred into corresponding wells in white and clear plates. The white plates were first left for 30 min pre-incubation and then 50 μL pseudotyped virus (100 ng p24/mL) was added per well, and the plates further incubated for 2 days. A volume of 50 μL culture medium per well was added to the clear plates (for the MTT assay) and cultured 2 days for cell viability assessment. For luciferase reporter assays, cells were lysed in 1X Glo Lysis Buffer (Promega). The luciferase activity was assessed by bioluminometer using the Bright-Glo luciferase assay system (Promega) according to the manufacturer's instructions. Cell viability was assayed by the MTT method. Cell viability and pseudo typed virus infectivity were calculated as percentages of the control without any added compound.

For determining $CC_{50}$ and $EC_{50}$, compounds were serially diluted and assayed as described for the HTS screening, $CC_{50}$ and $EC_{50}$ were calculated using 4 parameter regression.

#### 4.3.10. Statistical analysis

Statistical analyses were conducted using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA) software. The values of $CC_{50}$ and $IC_{50}$ were estimated using the log (inhibitor) versus normalized response nonlinear fit.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2021.105346.

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