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Synthesis and evaluation of enantiomers of hydroxychloroquine against SARS-CoV-2 in vitro

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ARTICLE INFO

Keywords:
Hydroxychloroquine
Enantiomers
Antiviral
SARS-CoV-2
COVID-19

ABSTRACT

Since the end of 2019, the outbreak of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has evolved into a global pandemic. There is an urgent need for effective and low-toxic antiviral drugs to remedy Remdesivir’s limitation. Hydroxychloroquine, a broad spectrum anti-viral drug, showed inhibitory activity against SARS-CoV-2 in some studies. Thus, we adopted a drug repurposing strategy, and further investigated hydroxychloroquine. We obtained different configurations of hydroxychloroquine side chains by using chiral resolution technique, and successfully furnished R-/S-hydroxychloroquine sulfate through chemical synthesis. The R configuration of hydroxychloroquine was found to exhibit higher antiviral activity (EC50 = 3.05 μM) and lower toxicity in vivo. Therefore, R-HCQ is a promising lead compound against SARS-CoV-2. Our research provides new strategy for the subsequent research on small molecule inhibitors against SARS-CoV-2.

1. Introduction

East Respiratory Syndrome (MERS), Severe Acute Respiratory Syndrome (SARS) and Ebola virus have caused a large number of infections and deaths. Most recently, at the end of 2019, a new type of coronavirus,¹ severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2), its genome sequence is highly consistent with SARS-CoV,² suddenly appeared.³ Due to its strong contagiousness (especially for elderly people,⁴ people with underlying diseases and immunocompromised individuals), this virus is raging widely and developed into a global pandemic. As the virus spreads, several mutated strains have been reported,⁶,⁷ which may cause easier human-to-human transmission a higher mortality rate before effective vaccines and therapeutic drugs are available. So far, there are more than 240 million people infected by coronavirus disease 2019 (COVID-19) with nearly five million recorded deaths worldwide.⁸ It highlights the challenges governments and medical institutions face in responding to sudden outbreaks. Therefore, there is an urgent need to develop highly effective drugs against novel coronavirus to protect people’s health.

Development of highly effective new drugs for a new virus is costly and time-consuming. Although many small molecule inhibitors against different targets of SARS-CoV-2 have been discovered,¹⁰,¹¹ most of them have only undergone partial preclinical or clinical research,¹²–¹⁸ and whether they can be formulated into drugs remains to be determined. In the face of a rapidly spreading and increasingly serious epidemic, these inhibitors cannot solve the current dilemma immediately.

In light of the challenges, drug repurposing may be an ideal strategy. Drug repurposing has many advantages¹⁹–²¹ including saving time, money, and the guarantee of safety with clinical experiences. There are many successful applications of this strategy in the past, such as thalidomide, ketoconazole, finasteride and so on. The World Health Organization launched a multinational randomized trial called “the solidarity trial” in 2020 to study the efficacy of some promising molecules or drug combinations against COVID-19.²² Its subset includes

Abbreviations: SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; MERS, Middle East Respiratory Syndrome; COVID-19, coronavirus disease 2019; remdesivir-TP, remdesivir triphosphate complex; FDA, U.S. Food and Drug Administration; CPE, cytopathic effect; dpd, day post drug administration; r.t., room temperature.

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https://doi.org/10.1016/j.bmc.2021.116523
Received 12 July 2021; Received in revised form 7 November 2021; Accepted 16 November 2021
Available online 22 November 2021
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Removal of viral RNA, we further tested the level of viral RNA in the cell supernatant. Consistent with the above results, all three drugs showed effective inhibition on the replication of the virus (Fig. 3B). Among them, R-HCQ exhibited strongest inhibitory activity (EC_{50} = 3.05 μM), while the inhibitory levels of hydroxychloroquine in racemate (EC_{50} = 5.09 μM) and S configuration (EC_{50} = 5.38 μM) were comparable. These data

2. Results

2.1. Synthesis

The synthetic routes of (S)-hydroxychloroquine sulfate and (R)-hydroxychloroquine sulfate were depicted in Schemes 1. The synthesis began with the recrystallization of commercially available starting materials 5 with mandelic acid of different configurations as reported methods. Chiral amine 8 or 9 was prepared from the mandelate 6 or 7 using sodium hydroxide to remove the corresponding mandelic acid. Subsequently, treatment of the amine with 4,7-dichloroquinoline gave compound 3 and 4, respectively, with good yields, followed by reaction with sulfuric acid furnished the final products hydroxychloroquine sulfate 10 and 11.

In order to test the purity and enantiomeric excess values of the asymmetric synthesis of hydroxychloroquine, we performed a new chiral HPLC analysis method, and the results are shown in Fig. 2 and Fig. S1 in Supporting information, SI. The purity of hydroxychloroquine exceeded 95%, and the enantiomeric excess values of hydroxychloroquine were all higher than 99%. The results confirm that we had indeed obtained a pair of enantiomers with high purity.

2.2. Preliminary biochemical evaluation of hydroxychloroquine and its Enantiomers

In order to evaluate the inhibitory effect of hydroxychloroquine and its enantiomers on the cytopathic effect (CPE) caused by the new coronavirus infection, we conducted an observational assay on the correlation between drug treatment and CPE. As shown in Fig. 3A, obvious cytopathic changes occurred after Vero E6 cells were infected with SARS-CoV-2, while Remdesivir treatment completely inhibited the occurrence of lesions. The drug treatment group (racemic hydroxychloroquine, R-Hydroxychloroquine sulfate and S-hydroxychloroquine sulfate) also showed complete inhibition of cytopathic changes caused by the virus at 40 μM. These results indicated that the enantiomers of hydroxychloroquine had inhibitory activity against SARS-CoV-2.

To examine the effect of these three compounds on the removal of viral RNA, we further tested the level of viral RNA in the cell supernatant. Consistent with the above results, all three drugs showed effective inhibition on the replication of the virus (Fig. 3B). Among them, R-HCQ exhibited strongest inhibitory activity (EC_{50} = 3.05 μM), while the inhibitory levels of hydroxychloroquine in racemate (EC_{50} = 5.09 μM) and S configuration (EC_{50} = 5.38 μM) were comparable. These data

Fig. 1. Representative antiviral drugs and enantiomers of hydroxychloroquine.
implied that R-HCQ might be the most active one of the enantiomers of hydroxychloroquine against SARS-CoV-2.

2.3. In vivo acute toxicity evaluation

Having obtained in vitro antiviral activities of these three compounds, we further evaluated their toxicity in vivo in a double-dose acute toxicity assay. When administered orally at a dose of 230 mg/kg with the compounds, the mice treated with Rac-HCQ, R-HCQ and S-HCQ behaved differently. As described in Fig. 4, the mice of the racemate group died at the 7th day post drug administration (dpd), and the mice of the S configuration group appeared malaise at 9 dpd, while the mice of the R configuration group performed well until the 12 dpd. More mice died after 15 dpd in the racemate group and S configuration group, and relatively stable number still maintained in R configuration group. Hence, the compound R-HCQ could be better tolerated in mice when compared to Rac-HCQ and S-HCQ.

3. Discussion

The outbreak of SARS-CoV-2 has evolved into an emergent global pandemic. Although Remdesivir has been approved by the FDA as the first drug for the treatment of COVID-19, its effectiveness is still limited and the treatment was accompanied by many side effects, such as gastrointestinal symptoms and heart and lung failure. More importantly, the clinical application of Remdesivir did not reduce the mortality caused by COVID-19. Therefore, it is still urgent to find effective and low-toxic antiviral drugs that can control the infection.

We adopted the idea of drug repurposing and focus on hydroxychloroquine. The data showed for the application of the racemate hydroxychloroquine as an anti-COVID-19 drug for clinical research is, at best, mixed. We speculated that the reason for the inconsistent clinical effects of hydroxychloroquine may be due to the lower activity or higher toxicity of one of its enantiomers, which affects the antiviral effect of the racemate. Therefore, we obtained hydroxychloroquine with different...
configuration of side chains by using the technique of chiral resolution, and successfully furnished R- and S-hydroxychloroquine sulfate through chemical synthesis. The pair of enantiomers with an enantiomeric excess greater than 99% were obtained and we further tested the inhibitory activity of racemic hydroxychloroquine, R configuration hydroxychloroquine, and S configuration hydroxychloroquine against SARS-CoV-2 virus in Vero E6 cells. In vitro CPE observation and viral RNA level detection assays showed that both racemic hydroxychloroquine and optically pure hydroxychloroquine have inhibitory but limited activity on SARS-CoV-2. When compared to Rac-HCQ, R-HCQ showed more efficient antiviral activity, while the activity of S-HCQ was comparable to that of the Rac-HCQ. We further conducted the acute toxicity studies in vivo, the toxicity of R-HCQ was lower than that of racemate and S-HCQ. We will conduct long-term toxicity assay on the two enantiomers to complete the toxicity studies, and test the antiviral activity of R-HCQ in vivo.

4. Conclusion

Through chiral resolution and chemical synthesis, we successfully synthesized optically pure hydroxychloroquine. Among them, R configuration hydroxychloroquine exhibited higher antiviral activity \((EC_{50} = 3.05 \, \mu M)\) than S configuration and racemic hydroxychloroquine. Acute toxicity tests in vivo showed that S-hydroxychloroquine had higher toxicity with racemates, while R configuration has lower toxicity. This is the first report that R-hydroxychloroquine has better in vivo toxicity than S-hydroxychloroquine. Based on the content above, the synthesis and biological functions of R-HCQ may be an important inspiration in promoting this field. Taken together, R-HCQ might be a promising lead compound for further investigation of antiviral drugs against SARS-CoV-2.

5. Materials and methods

5.1. Chemistry general methods

Reagents and solvents from commercial sources were used without further purification. The progress of all reactions was monitored by TLC using EtOAc/n-hexane or DCM/MeOH as solvent system, and spots were visualized by irradiation with UV light (254 nm) or staining with phosphomolybdic acid. Flash chromatography was performed using silica gel (300–400 mesh). \(^1\)H NMR and \(^{13}\)C NMR spectra were recorded on a Bruker Avance ARX-300 or a Bruker Avance ARX-400. Chemical shifts are reported in ppm, and multiplicity of signals are denoted as: s = singlet, d = doublet, t = triplet and m = multiplet. The low resolution ESIMS was recorded on an Agilent 1200 HPLC-MSD mass spectrometer and the high resolution on an Applied Biosystems Q-STAR Elite ESI-LC-MS/MS mass spectrometer. Anhydrous toluene and tetrahydrofuran (THF) were freshly distilled from sodium with benzophenone as the indicator. All other solvents were reagent grade. All moisture sensitive reactions were carried out in flame dried flask under argon atmosphere. The purity of the final compounds was determined by Agilent 1260.
series HPLC system using the following conditions: chiralpak IH30CE-WB024 column (DAICEL, 0.46 cm I.D. \times 25 cm \times 3 \mu m) with the solvent system (elution conditions: mobile phase A consisting of n-hexane/diethylamine with a ratio of 100/0.1; mobile phase B consisting of isopropanol/methanol/diethylamine with a ratio of 75/25/0.1), with monitoring 254 nm. A flow rate of 0.8 mL/min was used and the column temperature is 35 \degree C. The retention time was reported as \( t_R \) (min). The purity of final compounds is \( >95\% \).

5.2. Experimental procedures

(S)-2-((4-aminopentyl)(ethyl)amino)ethan-1-ol (S)- mandelate (6). A solution of 2-((4-aminopentyl)(ethyl)amino)ethan-1-ol (8.0 g, 45.9 mmol) in 2-propanol (100 mL) was added to a solution of S- (+)-mandelic acid (3.5 g, 23.0 mmol) in 2-propanol (20 mL). The mixture was stirred overnight at room temperature. Filtration gave white crystals which were recrystallized twice more from 2-propanol (100 mL and 80 mL respectively) to afford 6 (7.7 g, 52\%) as white crystals.

(R)-2-((4-aminopentyl)(ethyl)amino)ethan-1-ol (R)- mandelate (7). Compound 7 was prepared from 5 and R-mandelic acid in a similar manner as described for 6. White crystals. (8.1 g, 56\%).

(S)-2-((4-aminopentyl)(ethyl)amino)ethan-1-ol (8). To a solution of (S)-2-((4-aminopentyl)(ethyl)amino)ethan-1-ol (S)- mandelate (7 g, 21.6 mmol) in tert-butyl methyl ether (50 mL) was cooled to 0 \degree C. This clear solution was dropwise treated with 1 M NaOH aq. to adjust the pH of the mixture to 12. Upon complete addition, the reaction was warmed to r.t. over 2 h. The aqueous layer was extracted sextic with tert-butyl methyl ether (60 mL), and the organic layer washed with brine and dried over anhydrous Na2SO4, filtered and concentrated under reduced pressure to give the title compound (3.3 g, 83\%) as colorless oil, which was used in the next step without further purification.

1H NMR (300 MHz, Chloroform-d): \( \delta \) 3.52 (t, \( J = 5.4 \) Hz, 2H), 2.88 (h, \( J = 6.3 \) Hz, 1H), 2.60–2.49 (m, 4H), 2.44 (t, \( J = 7.3 \) Hz, 2H), 1.54–1.39 (m, 2H), 1.34–1.24 (m, 2H), 1.10–0.95 (m, 6H).

13C NMR (75 MHz, Chloroform-d): \( \delta \) 77.27, 58.33, 54.93, 53.28, 47.22, 46.78, 37.77, 24.17, 24.04, 11.80. HRMS (ESI+): \( m/z \) calculated for C9H23N2O (M + 1)+ 175.1805 found 175.1808.

(R)-2-((4-aminopentyl)(ethyl)amino)ethan-1-ol (9). Compound 9 was prepared from 7 in a similar manner as described for 8. Colorless oil (2.6 g, 80\%). 1H NMR (400 MHz, Chloroform-d): \( \delta \) 3.52 (t, \( J = 5.4 \) Hz, 2H), 2.87 (h, \( J = 6.3 \) Hz, 1H), 2.60–2.50 (m, 4H), 2.44 (t, \( J = 7.3 \) Hz, 2H), 1.54–1.39 (m, 2H), 1.34–1.24 (m, 2H), 1.10–0.95 (m, 6H).

Fig. 3. Preliminary biochemical evaluation of hydroxychloroquine and its enantiomers. A: CPE observation trial of Rac-HCQ sulfate, R-HCQ sulfate and S-HCQ sulfate using Remdesivir as a positive control; B: viral RNA level detection trial.

Fig. 4. The survival curve of mice in acute toxicity assay.
Y. Ni et al.
Bioorganic & Medicinal Chemistry 53 (2022) 116523

2H), 1.52–1.38 (m, 2H), 1.34–1.25 (m, 2H), 1.05 (d, J = 6.3 Hz, 3H), 1.00 (t, J = 7.1 Hz, 3H). \(^{1}H NMR\) (300 MHz, Deuterium Oxide): \(\delta\) 8.22 (d, J = 7.2 Hz, 1H), 8.11 (d, J = 9.0 Hz, 1H), 7.70 (d, J = 2.0 Hz, 1H), 7.51 (d, J = 9.0 Hz, 1H), 6.79 (d, J = 7.3 Hz, 1H), 4.14–4.02 (m, 4H), 3.82 (t, J = 5.2 Hz, 2H), 3.22 (q, J = 5.2, 4.7 Hz, 1H), 1.79 (s, 4H), 1.36 (d, J = 6.4 Hz, 3H), 1.25–1.17 (m, 3H). \(^{13}C NMR\) (75 MHz, Deuterium Oxide): \(\delta\) 155.28, 142.08, 139.11, 137.99, 137.12, 134.00, 128.11, 128.01, 125.86, 97.36, 83.67, 77.50, 55.94, 49.40, 48.18, 42.20, 18.10, 18.00. \([\alpha]_{D}^{20} = -13.1\) (c = 1, H2O).

(5) \(2-\{(4-((7-chloroquinolin-4-yl) amino) pentyl) (ethyl) amino\} ethan-1-ol\) (10). A solution of (S)-2-\{(4-((7-chloroquinolin-4-yl) amino) pentyl) (ethyl) amino\} ethan-1-ol (500 mg, 1.5 mmol) in EtOH (1.21 g, 7.0 mmol), 4,7-dichloroquinoline (1.1 g, 5.8 mmol), and potassium carbonate (400 mg, 2.9 mmol) were heat to 135 \(^{\circ}\)C for overnight without any solvent. After cooled down to room temperature. The mixture was extracted with DCM (3 \(\times\) 100 mL) and the combined organic layers were dried over anhydrous Na2SO4, filtered and concentrated under reduced pressure. The resulting residue was passed through a silica gel column chromatography (95% methanol in DCM) to provide the title compound (1.3 g, yield 68%) as colorless oil. \(^{1}H NMR\) (300 MHz, Chloroform-d): \(\delta\) 8.51 (d, J = 5.4 Hz, 1H), 7.94 (d, J = 2.2 Hz, 1H), 7.71 (d, J = 9.0 Hz, 1H), 7.35 (dd, J = 8.9, 2.2 Hz, 1H), 6.40 (d, J = 5.5 Hz, 1H), 4.96 (d, J = 7.7 Hz, 1H), 3.76–3.63 (m, 3H), 3.56 (t, J = 5.4 Hz, 2H), 2.61–2.54 (m, 4H), 2.50 (q, J = 4.4, 2.5 Hz, 2H), 1.75–1.52 (m, 4H), 1.32 (d, J = 6.3 Hz, 3H), 1.02 (t, J = 7.1 Hz, 3H). \(^{13}C NMR\) (75 MHz, Chloroform-d): \(\delta\) 151.87, 149.20, 149.03, 134.88, 128.68, 125.20, 121.13, 121.70, 99.13, 58.29, 54.90, 53.02, 48.39, 47.64, 34.16, 23.74, 20.41, 11.63. \([\alpha]_{D}^{20} = +95.8\) (c = 1, EtOH). HRMS (ESI): \(m/z\) calculated for C18H27ClN3O (M + 1)\(^{+}\) 336.1837 found 336.1841.

(4) \(2-\{(4-((7-chloroquinolin-4-yl) amino) pentyl) (ethyl) amino\} ethan-1-ol sulfate\) (10). White solid (232 mg, yield 67%).
this study were performed according to the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of China Pharmaceu-
tical University following the guidelines of the Association for
Assessment and Accreditation of Laboratory Animal Care (AAALAC).
The compounds Rac-HQCh sulfate, H-HQCh sulfate and S-HQCh sulfate were
dissolved in physiological saline. Sixteen male and 16 female mice (25 g) were
divided into four groups (n = 8): male and female control groups and male and female test groups. All mice were fasted overnight and
then administered intragastrically with the vehicle or 230 mg/kg of test
drugs twice a day. The death, daily behavior, and body weight of the mice were monitored during the subsequent 20 days.

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.

Acknowledgement

This work was supported by the National Natural Science Foundation (81773559), and the Double First-Class University Project
(CPU2018GY03) (S.) This work was also supported by Major science and technology project for the prevention and treatment of major
infectious diseases (2018ZX10301208), and National base cultivation project (20DZZ2210404).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.
onlinepageطول.2021.116523.

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