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Broad spectrum anti-coronavirus activity of a series of anti-malaria quinoline analogues

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ABSTRACT

In this study, a series of 10 quinoline analogues was evaluated for their in vitro antiviral activity against a panel of alpha- and beta-coronaviruses, including the severe acute respiratory syndrome coronaviruses 1 and 2 (SARS-CoV-1 and SARS-CoV-2), as well as the human coronaviruses (HCoV) 229E and OC43. Chloroquine and hydroxychloroquine were the most potent with antiviral EC\(_{50}\) values in the range of 0.12–12 \(\mu\)M. Chloroquine displayed the most favorable selectivity index (i.e. ratio cytotoxic versus antiviral concentration), being 165 for HCoV-OC43 in HEL cells. Potent anti-coronavirus activity was also observed with amodiaquine, ferroquine and mefloquine, although this was associated with substantial cytotoxicity for mefloquine. Primaquine, quinidine, quinine and tafenoquine only blocked coronavirus replication at higher concentrations, while piperaquine completely lacked antiviral and cytotoxic effects.

A time-of-addition experiment in HCoV-229E-infected HEL cells revealed that chloroquine interferes with viral entry at a post-attachment stage. Using confocal microscopy, no viral RNA synthesis could be detected upon treatment of SARS-CoV-2-infected cells with chloroquine. The inhibition of SARS-CoV-2 replication by chloroquine and hydroxychloroquine coincided with an inhibitory effect on the autophagy pathway as visualized by a dose-dependent increase in LC3-positive puncta. The latter effect was less pronounced or even absent with the other quinolines.

In summary, we showed that several quinoline analogues, including chloroquine, hydroxychloroquine, amodiaquine, ferroquine and mefloquine, exhibit broad anti-coronavirus activity in vitro.

1. Main text

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the cause of the ongoing coronavirus disease 2019 (COVID-19) pandemic, which is a worldwide challenge for health-care systems (Gorbalenya et al., 2020). As of April 4, 2021 more than 130 million cases and over 2.8 million deaths have been reported globally (WHO, 2021). COVID-19 is characterized by a mild to severe respiratory illness that appears to be influenced by age and comorbidities (Clark et al., 2020). The most frequent clinical presentation of severe COVID-19 is pneumonia with fever, cough and dyspnea (Merad and Martin, 2020). Today, seven human coronaviruses (HCoV) have been identified (Wang et al., 2020b). The highly pathogenic viruses, SARS-CoV-1 and Middle East respiratory syndrome coronavirus (MERS-CoV) belong, together with SARS-CoV-2, to the betacoronaviruses. On the other hand, the common cold coronaviruses, HCoV-229E and HCoV-NL63 (both alpha-coronaviruses), and HCoV-OC43 and HCoV-HKU1 (both betacoronaviruses) cause mild upper respiratory tract infections.

Coronaviruses are enveloped positive-sense RNA viruses, characterized by club-like spikes that protrude from their surface and an unusually large RNA genome (Perlman and Netland, 2009). The entry process is initiated by interaction of the viral spike protein with cellular receptors [e.g. angiotensin-converting enzyme 2 (ACE2) for SARS-CoV-2] (Wang et al., 2020a). These interactions, together with host factors (such as the cell surface serine protease TMPRSS2 or the endosomal cysteine protease cathepsin L), promote viral uptake and fusion at the cellular or...

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Fig. 1. Anti-coronavirus activity of chloroquine and its analogues. (A) Antiviral activity and toxicity of quinoline derivatives against alpha- and beta-coronaviridae assessed in HEL, Huh7 or Vero E6 cells. Antiviral activity is expressed as the 50% antiviral effective concentration (EC\textsubscript{50}), and cellular toxicity as the 50% cytotoxic concentration (CC\textsubscript{50}). Data shown are the mean ± SD of at least two independent tests performed in duplo; nd: not done. (B) In SARS-CoV-2-infected Vero E6 cells, hydroxychloroquine strongly decreased the release of virus particles in the supernatant, as measured by RT-qPCR at 48 h p.i. Data shown are the mean ± SEM of two independent experiments carried out in duplo; LOQ: limit of quantification. (C) Inhibitory effect of chloroquine on viral RNA synthesis in function of time of compound addition. HEL cells were infected with HCoV-229E virus, and compounds were added at 30 min, 0 h, 1 h, 3 h, 5 h or 8 h p.i. At 11 h p.i., the viral RNA was quantified by real-time RT-PCR. Data shown are the mean ± SEM of three independent experiments. (D) Effect of quinoline derivatives on the induction of cytoplasmic LC3 puncta. After incubation with the compounds for 3 h, cells were fixed, permeabilized and stained for LC3. Fluorescence was read on an ArrayScan XTI High Content Reader and the average pixel intensity was quantitated with the HCS Studio Software. Data shown are the mean ± SEM of three independent experiments.
endosomal membrane. Following release and uncoating of the genomic RNA, the viral replication and transcription complex is formed. Replication and transcription of the viral RNA take place in characteristic perinuclear double-membrane vesicles (V Kovski et al., 2020).

Due to the lack of effective antiviral drugs against coronaviruses, repurposing of clinically approved drugs for use as antivirals is an attractive strategy to cope with the COVID-19 pandemic. In this perspective, the quinoline derivatives chloroquine and hydroxychloroquine draw attention. Both drugs are being used for the prophylaxis and treatment of malaria, but also exert immunomodulatory effects (Savarino et al., 2003). In addition, they display broad spectrum antiviral activity against a range of diverse viruses, such as HIV-1 and HIV-2, Dengue virus, Zika virus, Chikungunya virus, Ebola virus, influenza A virus and herpes simplex virus type 1 (Boonyasuppayakorn et al., 2014; Delvecchio et al., 2016; Dowall et al., 2015; Khan et al., 2010; Ooi et al., 2006; Savarino et al., 2001; Singh et al., 1996). In 2004, Keyaerts et al. reported that chloroquine effectively inhibits SARS-CoV-1 in Vero E6 cells. Also, MERS-CoV, HCoV-229E and HCoV-OC43 were blocked by chloroquine in vitro (de Wilde et al., 2014; Keyaerts et al., 2009). In addition, chloroquine was highly effective against HCoV-OC43 infection in newborn mice. However, the antiviral effectiveness of chloroquine against SARS-CoV-2 was cell-type dependent, since it inhibited virus replication in Vero E6 cells, but not in Calu3 and Caco2 cells (Ellinger et al., 2021; Hoffmann et al., 2020). Hydroxychloroquine showed consistent ant-SARS-CoV-2 activity in Vero E6 and variable activity in Caco2 cells, but lacked antiviral activity in Calu3 cells (Clementi et al., 2020; Ellinger et al., 2021; Hoffmann et al., 2020). In vivo studies in preclinical animal models were disappointing since hydroxychloroquine conferred no protection against SARS-CoV-2 in macaques and a hamster model (Kaptein et al., 2020; Maisonnasse et al., 2020). Also, the overall conclusion of numerous clinical studies is that chloroquine treatment of SARS-CoV-2 infected patients is not beneficial (Horby et al., 2020). Recently, other anti-malarial quinoline analogues were reported to inhibit SARS-CoV-2 in Vero E6 cells (Gendrot et al., 2020). Mefloquine was also shown to be effective against SARS-CoV-2 in Caco2 cells and a hamster model (Ellinger et al., 2021).

However, a systematic side-by-side comparison of the antiviral activity of quinoline analogues against several human alpha- and beta-coronaviruses in different cell lines has not been reported. In this study, the FDA-approved antimalarial drugs chloroquine, hydroxychloroquine, amodiaquine, ferroquine, mefloquine, quinine, quinidine, piperaquine, primaquine and tafenoquine (see Supplementary Information for chemical structures) were investigated for their broad spectrum antiviral activity against HCoV-229E (an alphacoronavirus), HCoV-OC43, SARS-CoV-1 and SARS-CoV-2 (which are all betacoronaviruses). Antiviral assays were conducted in human embryonic lung fibroblasts (HEL 299), human hepatoblastoma cells (Huh7) and African green monkey kidney cells (Vero E6). The ProTide remdesivir, or its parent nucleoside GS-441524, were included as positive controls, because of their known broad spectrum anti-coronavirus activity (Sheahan et al., 2017). Briefly, the cells were infected with coronavirus in the presence of serial dilutions of the compounds and incubated for 3–4 days (Huh7 and Vero E6 cells) or 7 days (HEL cells) (see Supplementary Information for experimental details). In Huh7 and HEL cells, the virus-induced cytopathic effect was measured with the spectrophotometric formazan-based 3-(4,5-dimethylthiazol-2-y)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell viability assay after 7 days, the green fluorescent protein (GFP)-expressing Vero E6 cells allowed to evaluate the SARS-CoV-1- and SARS-CoV-2-induced cytopathic effect by measuring fluorescence at 4 days post infection (p.i.). The antiviral activity was expressed as the concentration producing 50% antiviral effect (EC50). In parallel, mock-infected cells were treated with serial dilutions of the compounds to evaluate their cellular toxicity, which was expressed as the 50% cytotoxic concentration (CC50).

The majority of the quinoline analogues did show antiviral activity against one or more coronaviruses tested. Only piperaquine completely lacked antiviral activity and cytotoxicity at the highest concentration tested (i.e. 100 μM). Among the quinolines tested, chloroquine and hydroxychloroquine were the most potent analogues with EC50 values in the range of 0.12–12 μM against the various coronaviruses (Fig. 1A). Especially, the activity of chloroquine against HCoV-OC43 in HEL cells is noteworthy with an EC50 value of 0.12 μM and a selectivity index (i.e. ratio of the cytotoxic concentration versus antiviral concentration) of 165. However, overall, the profile of hydroxychloroquine looked somewhat more attractive because of its lower cytotoxicity in the various cell lines, giving rise to more favorable selectivity indexes. Although mefloquine showed potent antiviral activity against the different coronaviruses, this was accompanied with substantial cytotoxicity in the HEL, Huh7 and Vero E6 cells. This is in agreement with previous findings, reporting cellular toxicity of mefloquine in Vero E6 cells (Gendrot et al., 2020), but in conflict with another paper (Jan et al., 2021) that showed a CC50 value for mefloquine in Vero E6 cells exceeding 100 μM. Amodiaquine and ferroquine displayed potent antiviral activity as well, and the lower cytotoxicity led to improved selectivity indexes. Also, primaquine, quinidine, quinine and tafenoquine displayed antiviral activity against selected coronaviruses. However, this was usually observed at higher concentrations (EC50 values exceeding 13 μM) with low selectivity indexes, indicating a lack of a selective antiviral effect.

A common feature of all compounds is the presence of a side chain, carrying a basic nitrogen, on a quinoline scaffold. This side chain is structurally diverse and can be acyclic [(hydroxy)chloroquine and primaquine], cycloaliphatic (quinine and quinidine) or aromatic (amodiaquine and ferroquine). However, a basic nitrogen is not sufficient for antiviral activity, since piperaquine is completely devoid of activity. Instead, piperaquine is the only bisquinoline analogue, whereas all other derivatives are based on a monoquinoline skeleton, suggesting that there are steric constraints.

The anti-SARS-CoV-2 activity of hydroxychloroquine in Vero E6 cells, as determined by high content imaging read-out, was confirmed by a virus yield assay. At 48 h p.i., the RNA levels of SARS-CoV-2 in the cell culture supernatants were determined by RT-qPCR (Fig. 1B). GS-441524 was included as positive control and produced a 4.3-log reduction of the viral copy number at 3.7 μM. Chloroquine caused a 2.9-log decrease in viral copy number at 1 μM, and even a 5.7-log decrease at 33 μM. This confirms that hydroxychloroquine is a potent inhibitor of coronavirus replication in vitro.

In order to delineate the step of the viral life cycle that is targeted by this compound class, we performed a time-of-addition experiment with chloroquine in HEL cells infected with HCoV-229E. Hepatoma hybrid agglutinin (HHA) and GS-441524 were included as reference compounds, as they have an established mode of action. Briefly, HEL cells were seeded in 48-well dishes at 40,000 cells per well, followed by overnight incubation at 37 °C. After a 1 h incubation on ice, the cells were infected with 30 CCID50 of the HCoV-229E virus and incubated at 35 °C. The test compounds were added at ~30 min, 0 h, 1 h, 3 h, 5 h or 8 h p.i. At 11 h p.i., viral RNA was quantified by one-step qRT-PCR (see Supplementary Information for details). In the absence of compound, the viral RNA copy number increased by 20-fold in 11 h p.i. (Fig. 1C). HHA, which interferes with virus entry at a post-attachment step, needs to be added prior to the virus (van der Meer et al., 2007), and quickly loses its inhibitory effect when added post infection. Instead, 8 h p.i., the parent nucleoside GS-441524 blocks the viral RNA synthesis (Vin et al., 2020), and thus can be administered up to 3 h p.i. Chloroquine completely blocked viral RNA synthesis when added at 1 h p.i., but gradually showed a diminished antiviral efficacy, at 3 h p.i. or later on. A similar time-dependency was reported for chloroquine in SARS-CoV-1 infected Vero E6 cells, while in HCoV-OC43-infected HRT-18G cells chloroquine lost its antiviral activity when added at 2 h p.i. (Keyaerts et al., 2004, 2009). Altogether, these data suggest that chloroquine interferes with the viral entry process at a post attachment.
Regarding its precise mechanism of action, several hypotheses have been proposed. Chloroquine has been reported to interfere with the terminal glycosylation of ACE2, the cellular receptor used by SARS-CoV-1 and SARS-CoV-2, resulting in a reduced binding of the viral spike protein to its receptor (Vincent et al., 2005). However, this hypothesis does not correspond with inhibition of the viral entry process at a post-attachment stage as shown in our time-of-addition experiments. On the other hand, the lysosomotropic properties of chloroquine result in an increased endosomal pH, and thus the potential to inhibit (corona)viruses that depend on low pH for activation, for example by cathepsin L (Ou et al., 2021; Tian et al., 2021). Chloroquine has also been reported to inhibit autophagy, which was assumed to result from its lysosomotropic properties. Though, Mauthe et al. showed that chloroquine decreases fusion of autophagosomes with lysosomes, thereby stalling the autophagic flux (Mauthe et al., 2018). The induction of autophagy upon coronavirus infection, indicates that coronaviruses employ components of the autophagic pathway. Hence, autophagy inhibition is a viable strategy for the development of anti-coronavirus agents. (Miller et al., 2020).

The combined effects of chloroquine on viral RNA replication and autophagy were visualized by confocal microscopy. Therefore, SARS-CoV-2- or HCoV-229E-infected Hu7 cells were treated with various concentrations of chloroquine (0.5, 5 and 50 μM) and incubated for 6 h, respectively (see Supplementary Information for details). Then, the cells were stained for double-stranded RNA (dsRNA) and LC3, markers for viral RNA replication and autophagy, respectively (Baggen et al., 2021; Tanida et al., 2008). The nuclei were visualized by fluorescent staining with 4,6-diamidino-2-phenylindole (DAPI).

In SARS-CoV-2-infected cells, viral RNA synthesis was completely blocked in the presence of chloroquine at 5 μM (Fig. 2). The higher concentration of chloroquine (i.e. 50 μM) required to fully inhibit HCoV-229E replication corresponds with its 4-fold higher antiviral EC50 for HCoV-229E compared to SARS-CoV-2 in Hu7 cells (Fig. 1A). Chloroquine treatment also resulted in a dose-dependent increase of LC3-positive puncta in infected and uninfected cells. High content imaging showed that chloroquine and hydroxychloroquine both produced a 6-fold increase of the intensity of cytoplasmic LC3 puncta, while for the other quinoline analogues only a moderate or no effect on LC3 staining was observed (Fig. 1D). This finding confirms the inhibitory effect of both chloroquine and hydroxychloroquine on the autophagy pathway and is in agreement with the fact that chloroquine and hydroxychloroquine are structurally very similar. In contrast, the other 8 analogues, although also based on a quinoline scaffold and having a basic side chain, are structurally more different and therefore it is not unexpected that they behave differently in various assays. These other quinoline analogues exert their antiviral effect probably via another mechanism of action that might be clinically more relevant.

In conclusion, chloroquine and several other quinolines efficiently inhibit the replication of a broad panel of alpha- and betacoronaviruses (including SARS-CoV-1 and SARS-CoV-2) in various cell types.

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 data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2021.105127.

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