The Effects of Chloroquine and Hydroxychloroquine on Nitric Oxide Production in RAW 264.7 and Bone Marrow-Derived Macrophages

(chloroquine / cytotoxicity / hydroxychloroquine / macrophages / NO production)

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Abstract. Chloroquine, an antimalarial drug, can also be used in the regulation of the immune system, e.g. it is used in the treatment of autoimmune diseases. In this study we investigated the effects of chloroquine and its hydroxy-derivative on nitric oxide (NO) production in two different cell types: (i) immortalized mouse macrophage cell line RAW 264.7 and (ii) mouse bone marrow-derived macrophages (BMDM). The cells were treated with different concentrations (1–100 µM) of chloroquine or hydroxychloroquine and stimulated with lipopolysaccharide for 24 h to induce NO production. Measurement of nitrites by the Griess reaction was used to evaluate the production of NO. Expression of inducible NO synthase was evaluated with Western blot and ATP-cytotoxicity test was used to measure the viability of the cells. Our results showed that both chloroquine and its hydroxy-derivative inhibited NO production in both cell types. However, based on the results of LD₅₀ these inhibitory effects of both derivatives were due to their cytotoxicity. The LD₅₀ values for chloroquine were 24.77 µM (RAW 264.7) and 24.86 µM (BMDM), the LD₅₀ for hydroxychloroquine were 13.28 µM (RAW 264.7) and 13.98 µM (BMDM). In conclusion, hydroxychloroquine was more cytotoxic than its parent molecule. Comparing the two cell types tested, our data suggest that there are no differences in cytotoxicity of chloroquine or hydroxychloroquine for primary cells (BMDM) or immortalized cell line (RAW 264.7).

Introduction

Chloroquine (CQ) was first synthesized 80 years ago and for decades has been the drug of choice in the treatment of malaria because of its efficiency and safety. The drug is administered orally. In malaria patients, CQ enters the digestive vacuole of the parasite inside the red blood cells and binds haem. This complex is very toxic and causes rupture of the vacuole and parasite death (Hempelmann, 2007). Nevertheless, due to its wide use, P. falciparum chloroquine resistance appeared several years after its introduction (Thome et al., 2013). Although the use of CQ is not indicated for malaria treatment in many countries, CQ was found to have immunomodulatory and anti-cancer effects (Solomon and Lee, 2009). CQ and its hydroxy-derivative hydroxychloroquine (HCQ) (Fig. 1) have been used in the treatment of autoimmune diseases, e.g. systemic lupus erythematosus or rheumatoid arthritis (Ben-Zvi et al., 2012).

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease characterized by bone erosion and cartilage damage with synovial hyperplasia and pain (Li and Wan, 2013). Macrophages, neutrophils and other immune cells are present in synovial tissue of RA patients and are involved in the development of RA pathogenesis. They produce inflammatory and degradative mediators that destruct the cartilage and bone (McInnes and Schett, 2011). Macrophages induce synovitis through release of cytokines, chemokines, reactive oxygen and nitrogen species (ROS/RNS), proteases and micropart-
cles (Mulherin et al., 1996). Singh et al. (2004) reported infiltration of macrophages into synovium at a very early stage of RA. The number of synovial macrophages correlates significantly with erosions (Mulherin et al., 1996; Bresnihan et al., 2007). Macrophages produce nitric oxide (NO) via inducible NO synthase (iNOS). ROS produced during oxidative burst of neutrophils may react with NO derived from macrophages to form RNS such as peroxynitrite (Hurst, 2012).

Despite the use of CQ and HCQ in the treatment of rheumatoid arthritis, the exact mechanism of immunosuppressive effects of these drugs is not known (Meier et al., 2013). It has been suggested that CQ and HCQ, which are weak lipophilic bases, diffuse into the lysosomes. Here they become protonated and unable to leave the lysosomes. This may interfere with the antigen presentation function. Lysosomal dysfunction leads to accumulation of debris and toxicity. Accumulation of ubiquinated proteins or impaired lysosomal functions may thus be related to CQ/HCQ-induced cell death. Therefore, induction of immune cell death or impaired activation of the immune system may be the modes of action of CQ and HCQ (Meier et al., 2013; Thome et al., 2013). On the other hand, there are few reports on the effects of CQ and especially HCQ on the production of NO in macrophages. Therefore, the aim of this study was to investigate the effects of CQ and HCQ on the production of NO in two different cell types: (i) immortalized RAW 264.7 mouse macrophage cell line, and (ii) mouse bone marrow-derived macrophages (BMDM). Inhibition of NO production in macrophages may contribute to resolution of inflammation.

Material and Methods

Bovine serum albumin (BSA), Griess reagent, and lipopolysaccharide (LPS) were purchased from Sigma (Schnelldorf, Germany). Anti-β-actin antibody was from Cell Signaling (Danvers, MA), anti-iNOS/NOS Type II mouse monoclonal antibody was from Bio-Rad (Hercules, CA), and ECL anti-mouse IgG HRP-linked whole antibody from eBiosciences (San Diego, CA). All other products are available commercially, or their origin is mentioned in the text.

RAW 264.7 cell culture

Murine peritoneal macrophage cell line RAW 264.7 (ATCC, Washington DC) was grown in Dulbecco’s modified Eagle’s medium (DMEM, PAN, Aidenbach, Germany) supplemented with 10% foetal bovine serum (FBS, PAN, Germany) and 1% gentamycin (Sigma-Aldrich, St. Louis, MO). Cells were maintained at 37 °C and 5% CO₂. After reaching confluence, the cells were harvested and used for the experiments.

Bone marrow-derived macrophages (BMDM)

Cells were isolated from mouse femurs and tibias by flushing the bone marrow with DMEM+10% FBS. The pooled cells were centrifuged (200 g, 10 min, RT) and resuspended and grown in DMEM media, which was supplemented with growth factors derived from cultured CCL-1 cells (ATCC, Manassas, VA), 20% of FBS and 1% gentamycin. The cells were left to adhere on the Petri dish during incubation in 37 °C and 5% CO₂ incubator. After reaching confluence, the cells were passaged. The experimental treatment was performed in DMEM+10 % FBS. BMDM were cultured no longer than 7 days before use.

Evaluation of nitrite production

Generation of reactive nitrogen species was determined indirectly as the accumulation of nitrates in the supernatant of RAW 264.7 or BMDM as described previously (Drabikova et al., 2010). Cells were incubated in 12-well plates (37 °C, 5% CO₂, 1 × 10⁶ cells/well) with or without CQ or HCQ (1-100 µM) and stimulated for 24 h with lipopolysaccharide (LPS, 10 ng/ml for RAW 264.7 or 100 ng/ml for BMDM). Samples treated with LPS only served as a positive control. At the end of the incubation, culture media were collected from the wells and centrifuged at 5000 g and 4 °C for 5 min. Then 150 µl of supernatant was mixed with an equal volume of Griess reagent in a 96-well plate, and the mixture was incubated at room temperature in the dark for 30 min. The absorbance was measured at 546 nm. Sodium nitrite was used as a standard.

Effect of CQ or HCQ on iNOS expression

The cell fractions from the previous protocol were used for the detection of iNOS expression by Western blot (Ambrozova et al., 2010). Briefly: cells were washed with cold PBS and lysed (1% SDS, 0.1 µM Tris pH 7.4, 10% glycerol, 1 µM NaVO₃, 1 µM phenylmethylsulfonyl fluoride). Protein concentrations were determined using BCA protein assay (Pierce, Rockford, IL), with BSA as a standard. Equal amounts of protein

Fig. 1. The structure of chloroquine (A) and hydroxychloroquine (B)
(10 µg/well) were loaded on 7.5% SDS-polyacrylamide gel for electrophoresis and then transferred to PVDF membrane. Anti-iNOS/NOS Type II mouse monoclonal antibody (1 : 2000) and ECL anti-mouse IgG HRP-linked whole antibody (1 : 2000) were used. The bands were detected using an ECL detection reagent kit (Pierce, USA) and exposed to the film (AGFA, Mortsel, Belgium). The equal loading of proteins was confirmed by determination of β-actin. The protein bands were measured densitometrically using ImageJ program and the individual band density value was expressed in arbitrary units.

**NO scavenging in a cell-free system**

The scavenging properties of CQ or HCQ against NO were measured amperometrically as described previously (Jancinova et al., 2010). A microsensor (AminiNO 700, Innovative Instruments, Lake Park Road Indian Trail, NC) was connected to the ISO-NO MARK II potentiostat (WPI, Sarasota, FL). Changes in electrical current of the control (PBS) or samples (PBS with CQ or HCQ at a final concentration of 100 µmol/l) were measured. The temperature was kept at 37 °C. The injection of NO-saturated water into the measurement glass vial (final concentration of NO = 2.38 µM) caused a rapid increase with a subsequent gradual decrease of the NO-induced signal until it reached the background current. The index of scavenging (the ratio of the signal peak height and the time from the NO injection to the re-entry of the signal to the background level) was used for evaluation of the scavenging properties of CQ and HCQ.

**Cell viability**

The cells were treated with or without CQ or HCQ (1–100 µM) for 24 h in white 96-well plates. After that, the supernatant was discarded and the remaining cells were lysed. ATP was determined by using luciferin/luciferase chemiluminescence assay. Non-treated cells served as a negative (viable) control. For positive (dead) control, saponin was added 15 minutes before the end of the incubation.

**Calculation of ED<sub>50</sub> and LD<sub>50</sub> values**

Based on the data of nitrite production or cytotoxicity of CQ and HCQ for each tested cell line we calculated the mean for each concentration used. Mean versus concentration data were plotted in a graph to create curves of ED<sub>50</sub> and LD<sub>50</sub>. The values of ED<sub>50</sub> and LD<sub>50</sub> were calculated from the linear part of the curves.

**Statistical analysis**

Data represent the mean ± SEM for four different experiments. Data were examined using the Student’s t-test and P values below 0.01 were considered statistically significant.

**Results**

As shown in Fig. 2 (A) and (B), both CQ and HCQ decreased the production of NO in RAW 264.7 and BMDM macrophages. Based on these data the calculated values of CQ ED<sub>50</sub> were 23.38 µM for RAW 264.7 cells and 30.25 µM for BMDM. For HCQ, the ED<sub>50</sub> values were 27.41 µM for RAW 264.7 cells and 31.43 µM for BMDM. These data clearly indicate similar efficiency of CQ and HCQ in decreasing the NO production. On the other hand, there is a mild increase in the concentration needed to reach ED<sub>50</sub> comparing the two different cell types.

As proved by amperometrical analysis, the decrease in NO production was not due to the scavenging of NO with CQ or HCQ. When using 100 µM concentrations of CQ and HCQ, the values were 100.84 ± 0.81 % and 101.96 ± 2.96 % of control, respectively.

The effects of CQ and HCQ on the viability of RAW 264.7 and BMDM macrophages are shown in Table 1. The LD<sub>50</sub> values for CQ were 24.77 µM (RAW 264.7) and 24.86 µM (BMDM), the LD<sub>50</sub> for HCQ were 13.28 µM (RAW 264.7) and 13.98 µM (BMDM). The correlation coefficients between inhibition of NO production and cytotoxicity in RAW 264.7 cells were 0.9809 for CQ (P < 0.0032) and 0.9665 for HCQ (P < 0.0073). Similarly, the correlation coefficients between
inhibition of NO production and cytotoxicity in BMDM cells were 0.9935 for CQ (P < 0.0006) and 0.9779 for HCQ (P < 0.0039). The decrease in chemiluminescence signal in the ATP assay was not due to the scavenging effects of CQ and HCQ, as tested in the cell-free assay (data not shown).

CQ and HCQ in concentrations that were below LD50 (1 and 10 µM) did not decrease the expression of iNOS measured by Western blot (data not shown).

### Discussion

Inducible NO synthase (iNOS) belongs to the NOS family, which is responsible for production of NO. Although iNOS was originally isolated from activated macrophages, its expression is induced in many cell types. The NO production by iNOS is responsible for bacterial killing in macrophages. On the other hand, it has also been implicated in many inflammatory diseases with autoimmune background (e.g. vasculitis, lupus, rheumatoid arthritis) (Pacher et al., 2007; Robinson et al., 2011). Different agents have been shown to decrease the activity of iNOS and thus the production of NO in macrophages (Djoko et al., 2007; Pekarova et al., 2009). In this study, we discuss the effects of two antimalarial agents – CQ and HCQ – on the production of NO in two types of macrophages: the immortalized RAW 264.7 cell line and macrophages derived from mouse bone marrow.

There are few reports showing the effects of CQ, and especially HCQ, on the NO production in macrophages. Hrabak et al. (1998) showed that CQ inhibited production of NO in mouse and rat peritoneal macrophages stimulated with interferon γ for 24 or 48 h already at 1 µM concentration. Vuolteenaho et al. (2005) compared HCQ with other disease-modifying anti-rheumatic drugs and showed that HCQ decreased production of NO in chondrocytes. Both authors used different stimuli and incubation times. We compared the effects of CQ and HCQ in the same conditions and we showed that there were no differences between the ED50 values of CQ and HCQ corresponding to RAW 264.7 or BMDM cells. We suggest that the addition of a hydroxyl group into the molecule of CQ has no additional benefits regarding the efficiency of NO production in macrophages.

CQ and HCQ are well-tolerated drugs, but the patients need to be monitored because of reported side effects. Both antimalarials are known for retinopathy and gastrointestinal irritation. However, fewer patients receiving HCQ have reported the side effects, suggesting HCQ to be safer in comparison with CQ. These side effects are usually mild and could be managed by dose reduction (Martínez-Costa et al., 2013; Rodriguez-Caruncho and Marsol, 2014). To answer the question regarding the cytotoxicity we compared the effects of CQ and HCQ on the viability of RAW 264.7 and BMDM macrophages. Comparing the LD50 values, in our experiments HCQ was more cytotoxic than its parent drug. In the experiments of Hrabak et al. (1998) and Vuolteenaho et al. (2005), the authors did not report any toxic effects of CQ and HCQ. This may be due to the short time of incubation and methods used to determine the toxic effects of CQ and HCQ. Both authors used spectrophotometric assays. We used the chemiluminescence assay, which detects ATP levels. By using spectrophotometric assay detecting the release of lactate dehydrogenase we found that CQ and HCQ may interfere with the assay (unpublished data). Moreover, microscopy examination of the cells treated for 24 h with higher concentrations of CQ and HCQ (25, 50 and 100 µM) showed dead cells and debris (unpublished data). The results of Hrabak et al. (1998) and Vuolteenaho et al. (2005) and our data suggest that the kinetics of NO down-regulation and cytotoxicity might be very important in evaluating the effects of CQ and HCQ on macrophages. Incubation between 1–24 h should be tested in more detail.

Finally, we compared the effects of CQ and HCQ on NO production in two different macrophage cell types – the artificial immortalized RAW 264.7 cell line, which is widely used in studies of the effects of different compounds on NO production and iNOS expression, and macrophages derived from mouse bone marrow. Moreover, based on the literature reporting anti-cancer effects of CQ (Fan et al., 2006; Zheng et al., 2009), we tested the hypothesis that the immortalized cell line might be more susceptible to cell death after treatment with CQ or HCQ in comparison with the isolated macrophages. Interestingly, our results showed that there were no significant differences in the effects of CQ and HCQ on the NO production between RAW 264.7 and BMDM cells. Similarly, LD50 values for CQ or HCQ were nearly the

### Table 1. The effects of CQ and HCQ on the viability of RAW 264.7 and BMDM macrophages

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>RAW</th>
<th>BMDM</th>
<th>RAW</th>
<th>BMDM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CQ</td>
<td>HCQ</td>
<td>CQ</td>
<td>HCQ</td>
</tr>
<tr>
<td>1</td>
<td>23.78 ± 9.98</td>
<td>37.27 ± 7.83</td>
<td>24.64 ± 10.78</td>
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<tr>
<td>10</td>
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<tr>
<td>25</td>
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<td>54.22 ± 7.11</td>
<td>55.67 ± 11.15</td>
<td>57.07 ± 10.61</td>
</tr>
<tr>
<td>50</td>
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<td>91.76 ± 2.27</td>
<td>85.56 ± 4.93</td>
</tr>
<tr>
<td>100</td>
<td>99.68 ± 0.03</td>
<td>99.65 ± 0.03</td>
<td>99.73 ± 0.02</td>
<td>99.73 ± 0.01</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SEM, N = 4. Values are shown as the percentage of dead cells in comparison with non-treated control.
same for RAW 264.7 cells and BMDM, showing that there were no differences in the induction of cell death between RAW 264.7 cells and BMDM. In the following experiments, we want to investigate the cytotoxic effects of CQ and HCQ at different time intervals and distinguishing the type of cell death.

CQ and HCQ are administered orally, usually in a single dose of 250-400 mg/day. These doses could be doubled in the first two weeks of the drug administration. However, this depends on whether malaria or rheumatoid disease is the case. Based on the pharmacokinetic information of the European Medicines Agency (EMA, 2013) and Medicines and Healthcare products Regulatory Agency in United Kingdom (MHRA, 2014) the single dose of 300 mg CQ or 400 mg of HCQ lead to the plasma concentrations of 0.2 µM and 0.3 µM, respectively (French et al., 1987; Maitland et al., 1997; EMA, 2013; MHRA, 2014). In comparison with our results, these plasma concentrations are 43–125-fold lower than LD₅₀ calculated from our experiments. On the other hand, these results suggest the use of concentrations 0.1–10 µM of CQ and HCQ in further experiments.

In conclusion, in our experiments the parent drug – CQ – had similar efficiency in the NO production in macrophages compared to its hydroxycoumarin-derivative. Moreover, CQ showed lower cytotoxicity than HCQ in the two studied cell lines. We found no difference between the RAW 264.7 cell line and BMDM cells regarding the cytotoxic effects of the tested compounds.

References


