Antimalarial drug chloroquine counteracts activation of indoleamine (2,3)-dioxygenase activity in human PBMC

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1. Introduction

Since the 1930s, chloroquine [(RS)-N'-(7-chloroquinolin-4-yl)-N,N-diethyl-pentane-1,4-diamine] is a widely used antimalarial drug, mainly due to its relatively good tolerability and the cost-efficient synthesis [1,2]. Upon cellular uptake, chloroquine accumulates in acidic organelles such as endosomes, lysosomes and in Golgi vesicles, where it interferes with the activity of enzymes and posttranslational protein modification steps [3,4]. Furthermore, drug accumulation decreases the intracellular iron concentration and induces oxidative stress [5,6]. Accumulated in the digestive vacuoles of parasite affected cells, chloroquine inhibits the malaria parasite’s digestive pathway for hemoglobin [7]. The development of resistant Plasmodium strains has now led to the replacement of the first-line treatment and prophylaxis with chloroquine and its derivatives with other therapeutics such as artemisinin [8,9]. However, chloroquine and analogs became interesting as treatment options for other immune-related disorders, due to their immunomodulatory properties, e.g. by interfering with pro-inflammatory cytokine secretion [10,11]. On the basis of its anti-inflammatory properties, chloroquine and hydroxychloroquine are used for the treatment of rheumatoid arthritis, discoid lupus erythematosus, amebic hepatitis and chronic Q fever [2,12–15].

The pro-inflammatory cytokine interferon-γ (IFNγ) plays a central role in the cellular immune response, as it induces several immune-regulatory pathways and cellular responses [16,17]. Inflammation is further characterized through the activation of the redox-sensitive transcription factor nuclear factor-kappa B (NF-kB). NF-kB regulates a variety of genes that control immune responses such as the pro-inflammatory cytokines [18]. IFNγ stimulates also the production of neopterin by guanosine triphosphate (GTP)-cyclodihydrolase-I (GTP-CH-I, EC 3.5.4.16) in macrophages [19]. Likewise, neopterin was found to support oxidation processes by reactive oxygen and chloride metabolites [20] as well as their formation in inflammatory cells like neutrophils [21]. In turn, in target cells redox-sensitive signaling cascades such as nuclear factor-κB are triggered by neopterin [22]. IFNγ signaling is also involved in the activation of indoleamine 2,3-dioxygenase (IDO, EC 1.13.11.52), the enzyme that catalyses the rate-limiting step in the conversion of tryptophan to kynurenine [23]. Neopterin levels, tryptophan concentrations and IDO activity have been successfully used to monitor cell-mediated immune activation and to reveal prognostic information in a variety of diseases, including rheumatoid arthritis [24–26].

Elevated concentrations of urine and serum neopterin have been detected in patients infected with Plasmodium falciparum and Plasmodium vivax from epidemiologically distinct populations [27–30]. Similarly, increased breakdown of tryptophan has been reported in a murine malaria model [31].

A more detailed analysis of potential interactions of chloroquine with interferon-γ-induced tryptophan breakdown and neopterin...
production might help to explain the beneficial effects for the treatment of rheumatic diseases and might introduce new therapeutic regimen for disorders that are associated with increased immune activation.

Therefore, the aim of this in vitro study was to investigate the immunomodulatory properties of chloroquine in human peripheral blood mononuclear cells (PBMC) and in THP-1-Blue cells. Mitogen-stimulated PBMC represent a widely used model to evaluate pro- and anti-inflammatory properties of compounds, where neopterin production and tryptophan degradation can be used as read-outs [32]. The THP-1-Blue cell line is transfected with a NF-κB/AP-1-inducible reporter system that allows the monitoring of NF-κB activity in cell supernatants. In this cell line, lipopolysaccharide (LPS)-induced NF-κB expression has been reported to correlate with neopterin production and IDO activity [33]. Further, the production of soluble interleukin 2 receptor alpha (sIL-2Rα) was used to monitor the influence of chloroquine on the inflammatory process [32,34].

2. Materials and methods

2.1. Chemicals

Lipopolysaccharide (LPS) of *Escherichia coli*, phytohemagglutinin (PHA) and chloroquine were obtained from Sigma–Aldrich (Vienna, Austria). The latter was dissolved in RPMI 1640 medium (MedPro, Vienna, Austria) before each experiment.

2.2. Isolation and culture of human PBMC

PBMC were isolated from whole blood obtained from healthy donors by density centrifugation (Lymphoprep, Nycomed Pharma AS, Oslo, Norway). After isolation, PBMC were washed three times in phosphate buffered saline containing 0.5 mM EDTA. Cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS, Biochrom, Berlin, Germany), 2 mM L-glutamine (Serva, Heidelberg, Germany) and 50 μg/ml gentamicin (Bio-Whittaker, Walkersville, MD) at 37 °C with 5% CO2.

2.3. THP-1-Blue cell culture

THP-1-Blue cells (Invivogen, San Diego, USA) were incubated at 37 °C with 5% CO2 in RPMI 1640 medium supplemented with 10% FCS and 200 μg/ml zoecon (Invivogen, San Diego, USA).

2.4. Cell treatment

PBMC were seeded at a density of 1.5 × 10^6 cells/ml in supplemented RPMI 1640, preincubated for 30 min with or without different concentrations of chloroquine (2.0–50 μM) and stimulated, or not, with 10 μg/ml PHA for another 48 h. THP-1-Blue cells were seeded at a density of 5 × 10^5 cells/ml in supplemented RPMI 1640. Cells were preincubated for 30 min with different concentrations of chloroquine (6.25–50 μM) and stimulated or not with 1 μg/ml LPS.

Supernatants for Kyn/Trp determination were collected after 48 h, after this period the accumulated tryptophan breakdown and neopterin formation reaches a plateau [32]. Supernatants from THP-1-Blue cells for NF-κB activity measurement were collected for both, 24 and 48 h treatment duration. However, in agreement with earlier observations, the read-out at 24 h was able to better discriminate results obtained with different concentrations of compounds [33].

### Table 1

| Concentrations of tryptophan, kynurenine, kynurenine to tryptophan ratio (Kyn/Trp) and neopterin in the supernatant of unstimulated PBMC and in cells stimulated with 10 μg/ml PHA for 48 h. Results shown are the mean values ± SEM of three independent experiments run in duplicates. |
|-----------------|-----------------|-----------------|
| **Kyn/Trp (μmol/mmol)** | **Neopterin (nM)** |
|-----------------|-----------------|
| Unstimulated PHA (10 μg/ml) | 1.9 ± 1.1 | 14.3 ± 0.7 | 12408 ± 4379 |
| 1.9 ± 1.1 | 14.3 ± 0.7 | 12408 ± 4379 |

* p < 0.05, compared to unstimulated cells.

2.5. Measurement of tryptophan, kynurenine, neopterin and sIL-2Rα concentrations

Tryptophan, kynurenine, neopterin and cytokine measurements were performed in centrifuged supernatants. Tryptophan and kynurenine concentrations were measured by high performance liquid chromatography (HPLC) using 3-nitro-l-tyrosine as an internal standard [35]. To estimate IDO activity, Kyn/Trp was calculated and expressed as μmol kynurenine/mmol tryptophan.

Neopterin concentrations were determined by ELISA (BRAHMS, Henningsdorf, Berlin, Germany) with a detection limit of 2 nM, IL-2Rα concentrations were measured by ELISA obtained from R&D (Biomedica, Vienna, Austria), both according to the manufacturer’s instructions.

2.6. Measurement of NF-κB activity and cell viability in THP-1-Blue cells

THP-1-Blue cells are stably transfected with a reporter plasmid expressing the secreted embryonic alkaline phosphatase (SEAP) under the control of a NF-κB/AP-1 inducible promoter. Upon NF-κB activation, SEAP is expressed and released into the media, where the enzyme activity has been measured in a colorimetric assay at 635 nm (Bio-Tek Instruments, Bad Friedrichshall, Germany) by incubating 10%(v/v) of cell supernatant with 90%(v/v) of Quanti-Blue reagent (Invivogen, San Diego, USA).

Cell viability was measured by CellTiter-Blue® assay (Promega, Vienna, Austria) according to the manufacturer's instructions.

2.7. Statistical analysis

For statistical analysis, the Statistical Package for the Social Sciences (version 19, SPSS, Chicago, Ill, USA) was used. Because not all data sets showed normal distribution, for comparison of grouped data non-parametric Friedman test and Wilcoxon signed-ranks test were applied. p-Values below 0.05 were considered to indicate significant differences.

3. Results

3.1. Effect of chloroquine on tryptophan metabolism and neopterin formation in PBMC

After an incubation period of 48 h, neopterin concentrations (± SEM) in culture supernatants of unstimulated PBMC were 3.8 ± 0.1 nM and the mean Kyn/Trp ratio was 99.2 ± 46.6 μmol/mmol. Upon stimulation of cells with the phytohemagglutinin (PHA), neopterin production increased to 20.1 ± 1.4 nM and the mean Kyn/Trp was increased approximately 100-fold. The concentrations of neopterin, tryptophan, kynurenine and IDO activity, indicated by Kyn/Trp, in unstimulated and PHA-stimulated PBMC are listed in Table 1.
Fig. 1. Effect of 48 h of chloroquine treatment on tryptophan (A) and kynurenine (B) concentrations, on IDO activity expressed as kynurenine to tryptophan ratio (Kyn/Trp) (C) and on neopterin production (D) in unstimulated (white bars) and PHA-stimulated PBMC (black bars), expressed as % of baseline (control cells treated with or without PHA, respectively). Results shown are the mean values ± SEM of three independent experiments run in duplicates (*p < 0.05, compared to cells without added chloroquine).

In unstimulated cells, tryptophan concentrations were not affected by chloroquine addition (2.0–50 μM of chloroquine, Fig. 1A), but kynurenine levels decreased in a dose-dependent manner (Fig. 1B). In PHA-stimulated PBMC, chloroquine suppressed tryptophan breakdown in a dose-dependent manner (Fig. 1A) and in parallel kynurenine levels declined (Fig. 1B). A reduction of the Kyn/Trp upon chloroquine treatment was dose-dependent in both, unstimulated and PHA-stimulated cells, with a more pronounced effect in PHA-stimulated cells (Fig. 1C).

The addition of chloroquine to unstimulated PBMC resulted in an only slight suppression of neopterin concentrations in culture supernatants, e.g. 20 μM chloroquine suppressed neopterin production (± SD) to 93.8 ± 3.5% of baseline. In PHA-stimulated cells, the neopterin formation was significantly increased at a concentration of 10 μM chloroquine (124.5 ± 4.0%), while 50 μM of chloroquine resulted in a strong decrease of neopterin concentrations down to 29.0 ± 10.4% compared to PHA-stimulated control cells (Fig. 1D).

3.2. Effect of chloroquine on sIL2Rα secretion in PHA-stimulated PBMC

sIL2Rα concentrations in cell culture supernatants were increased more than 100-fold in PHA-stimulated PBMC in comparison to unstimulated cells (p < 0.005), additional chloroquine treatment (10 and 50 μM) reduced this effect in a dose-dependent manner (p < 0.05 and 0.005 for 10 and 50 μM chloroquine- and PHA-stimulated PBMC in comparison to PHA-stimulated control, details not shown).

3.3. Effects of chloroquine in THP-1-Blue cells

Cytotoxicity of chloroquine was evaluated in THP-1-Blue cells treated with increasing doses for 24 h, with or without additional stimulation by lipopolysaccharide (LPS). Treatment resulted in a dose-dependent decrease of cell viability with IC50 values of 63.16 μM in unstimulated and 54.35 μM in stimulated cells.

After an incubation of 48 h, the Kyn/Trp (± SEM) was significantly increased in LPS-stimulated THP-1-Blue cells (96.4 ± 12.6 μmol/mmol) in comparison to unstimulated cells (20.4 ± 1.1 μmol/mmol). There was no effect on Kyn/Trp upon treatment of THP-1-Blue cells with chloroquine for both, LPS-stimulated and unstimulated cells (data not shown).

Upon 24 h of LPS stimulation, the activation of NF-κB according to SEAP activity was increased (9.60 ± 1.18 (SEM)-fold) in comparison to unstimulated cells (p < 0.005). The additional treatment with chloroquine decreased SEAP activity in a dose-dependent manner in LPS-stimulated cells (SEAP activity expressed as fold of unstimulated control: 12.5 μM = 6.77 ± 0.84, 50 μM = 4.24 ± 0.63, p < 0.005), while unstimulated cells were not affected (Fig. 2).

4. Discussion

In this study, the capacity of chloroquine to modulate immune responses in human PBMC and in myelomonocytic THP-1-Blue cells was investigated in vitro. In PBMC, both stimulated or not with the
NF-κB and subsequent expression of pro-inflammatory genes [42]. Therefore, the effect of chloroquine on NF-κB activation remains elusive.

In conclusion, our in vitro study shows that chloroquine treatment results in the suppression of distinct mechanisms in mitogen-stimulated PBMC in comparison to toll like receptor (TLR) stimulated mononuclear THP1-Blue cells. Data indicates that chloroquine might have stronger influence on IDO activity by acting on cytokine secretion of T-cells than by acting on THP-1-Blue monocytes. Although THP-1-Blue cells are at an advanced stage of myelomonocytic development and their responsiveness to LPS has been extensively examined, they represent an undifferentiated phenotype [33,43]. The use of macrophages that have an exaggerated response to LPS and act differently than monocytes, might give more insight into chloroquine’s mode of action.

However, our findings might be of importance for the discussion about the use of chloroquine for the treatment of other disorders associated with overwhelming immune response. The application of chloroquine and its analogs has already been proved efficient in the treatment studies of autoimmune disease, e.g. in systemic lupus erythematosus [12,13]. Furthermore, there are clinical trials ongoing with chloroquine and its derivatives that explore their potential for the treatment of viral infections and cancer, with human immunodeficiency virus (HIV) infection being the most studied disease [44–47]. Our results support the view that a more detailed analysis of chloroquine activities in further in vitro and in vivo studies will be of central importance to explore additional potential therapeutic regimes in other chronic inflammatory conditions such as coronary heart disease or neurodegeneration.

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