available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Growth inhibitory and differentiation effects of chloroquine and its analogue on human leukemic cells potentiate fetal hemoglobin production by targeting the polyamine pathway

Efemwonkiekie Iyamu [a,b,*], *Harrison Perdew* [a], *Gerald Woods* [a,b]

[a] *Division of Hematology and Oncology, Children's Mercy Hospital & Clinics, Kansas City, MO 64108, United States*
[b] *Pediatrics, University of Missouri Medical Center at Kansas City, Kansas City, MO 64108, United States*

ARTICLE INFO

Article history:
Received 24 September 2008
Accepted 14 November 2008

Keywords:
Fetal hemoglobin
Inflammation
Erythroid differentiation
Chloroquine
Hydroxychloroquine
Arginase

ABSTRACT

Elevated arginase activity has been implicated in several pathological conditions in sickle cell disease (SCD) and other inflammatory disorders. Recently, we showed that chloroquine (CQ), an anti-malarial and anti-rheumatoid drug, displays a competitive mode of inhibition on sickle erythrocyte arginase. However, the effects of CQ and its analogue, hydroxychloroquine (HCQ) on erythroid differentiation leading to induced fetal hemoglobin (Hb F) production is unknown. In the present study, we obtained evidence of the anti-proliferative and differentiation effects of CQ and HCQ at pharmacologically attainable concentrations. This differentiation effect was linked to a dose-dependent inhibition of arginase activity and induced hemoglobinization, as Hb F synthesis was increased by 3.4- and 3.2-fold for CQ or HCQ, respectively. Treatment of K562 cells with lipopolysaccharide (LPS) or 8-bromo-cAMP (Br-cAMP) failed to reverse the inhibitory effects of CQ or HCQ on arginase activity. Indeed, the combination of Br-cAMP with CQ in LPS-treated cells resulted in a significant enhancement of Hb F and total hemoglobin production. Further, we showed that CQ or HCQ maximally stimulated intracellular cGMP levels by 6.6- and 3.0-fold at 6 and 3 h, respectively, as demonstrated by immunosorbent assay. However, co-treatment of K562 cells with CQ or HCQ in the presence of inhibitors of sGC-PKG-pathways reduced Hb F stimulation, suggesting the possible involvement of the sGC-PKG pathway. This is the first evidence demonstrating the capacity of anti-rheumatoid drugs to modulate the arginine-pathway and result in the enhancement of Hb F production, and thus may provide a paradigm for targeted therapy of hemoglobinopathies and other inflammation-related disorders.

1. Introduction

Human erythroleukemic K562 cells can be induced to differentiate by appropriate chemical agents, including hydroxyurea (HU), hemin butyric acid, 5-azacytidine, cytosine arabinoside, and cisplatin analogues, resulting in increased expression of fetal globin genes [1–7]. Therefore, the K562 cell line has been proposed as a very useful *in vitro* model system to determine the therapeutic potential of new differentiating compounds as well as to study the molecular mechanism(s) regulating changes in the fetal hemoglobin (Hb F) program [2,4]. Inducers of erythroid differentiation that

* *Corresponding author at*: Division of Hematology/Oncology, The Children's Mercy Hospital, Pediatric Research Center, 2401 Gillham Road, 3730.01 Kansas City, MO 64108, United States. Tel.: +1 913 302 1470; fax: +1 816 983 6515.
E-mail address: eiyamu@cmh.edu (E. Iyamu).
0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.11.016

stimulate Hb F production could be considered for possible use in the therapy of hemoglobinopathies such as sickle cell anemia, since the induction of Hb F in patients with sickle cell disease (SCD) has been shown to inhibits Hb S polymerization and ameliorates the overall clinical severity and mortality [8,9]. Of the known erythroid differentiation agents that stimulate Hb F synthesis, HU is currently the only FDA approved therapeutic drug available for the treatment and management of patients with SCD. But patients' response to this agent varies widely, and its oncogenic potential is also a major concern. Although the mechanism of Hb F-inducing action of HU is not clear, one of its mechanisms is presumed to involve the stimulation of nitric oxide (NO) synthase via the soluble guanylate cyclase-protein kinase G (sGC-PKG) signaling pathway [10,11].

Arginase (L-arginine amidinohydrolase, EC 3.5.3.1) is found predominantly in the liver and kidneys, but is also present in erythroid cells [12,13]. It can be induced in many cell types by a variety of cytokines and inflammatory stimuli [14,15]. Since arginine and ornithine compete for the same transport system for cellular uptake, a decrease in the ratio of arginine to ornithine resulting from increased arginase activity could limit arginine bioavailability for NO synthesis. This metabolic imbalance has been implicated in the clinical course of SCD as well as other inflammation-related disorders [16–18].

The anti-malarial drugs chloroquine (CQ) and its analogue, hydroxychloroquine (HCQ) have long been in use for the treatment of inflammatory diseases, such as rheumatoid arthritis and systemic lupus erythematosus (SLE) [19]. One of their mechanisms of action is thought to involve inhibition of T cell proliferation and suppression of the generation of immunoglobulin-secreting cells [20–23]. We recently observed that CQ directly inhibits arginase activity in sickle erythrocytes via a competitive mechanism [24]. However, knowledge of the link between the modulation of the arginase pathway and induced differentiation effects in K562 cells mediated by CQ or HCQ is unknown.

We have been studying the mechanisms of arginase modulation by anti-sickling drugs in vivo and in culture systems [24,25]. These studies have been extended in the present report to a consideration of the role of the inhibition of arginase activity by CQ or HCQ in the enhancement of K562 cells towards erythroid differentiation, and to elucidate the possible mechanism associated with said event.

Here we report for the first time, the results of the inhibition of arginase activity in cultured cells by CQ or HCQ. The inhibition of arginase activity by CQ or HCQ correlated with induced production of Hb F. The stimulation of arginase activity by LPS, or Br-cAMP failed to reverse the CQ- or HCQ-mediated inhibitory effects of arginase and the commitment of cells towards erythroid maturation. Also CQ exhibited induced cell differentiating effects in the presence of both spermine and spermidine; intermediates of polyamine pathway. To our knowledge, this is the first example of anti-malarial and anti-inflammatory agents displaying induced hemoglobinization in erythroid cells via the modulation of the polyamine pathway.

2. Materials and methods

2.1. Cell culture and reagents

Unless otherwise specified, all reagents and CQ, HCQ, 3-isobutyl-1-methylxanthine, Trypan blue solution, spermine, spermidine, DMSO and LPS were obtained from Sigma Chemical Co (St. Louis, MO, USA). The cAMP analog, LY83583, and KT5823 were obtained from EMD Biosciences (San Diego, CA, USA). The K562 cell line, derived from human erythroleukemic cells, was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in 25-cm^3 flasks in 10-ml medium consisting of RPMI 1640 (Gibco, Grand Island, NY, USA), containing 10% fetal bovine serum (FBS) and 50 U/ml of streptomycin–penicillin all obtained from Atlanta Biologicals (Atlanta, GA, USA). The cultures were maintained at 37 °C in 5% CO_2 atmosphere. Under these conditions, cell viability was greater than 93% as determined by Trypan blue exclusion.

2.2. Chloroquine or hydroxychloroquine treatment and inhibition studies

K562 cells were seeded in 25-cm^3 flasks at a density of 1×10^6 cells/ml and incubated in culture media containing various concentration of CQ or HCQ (0–100 μM) at 37 °C for 72 h. The 72-h incubation time was chosen for further experiments in this study because preliminary results indicated that at this end point, CQ or HCQ maximally induced K562 cells towards erythroid differentiation. The cell number was counted by Trypan blue exclusion assay. Experiments for each condition were performed in duplicate fashion. The IC_{50} value of each concentration of drug was calculated using the method of Chou and Talalay [26]. In another experiment, cells were treated with 5 μM spermine, 5 μM spermidine (intermediates of polyamine pathway), 10 μg/ml LPS, or 15 μM bromo-cAMP (Br-cAMP) in the presence of 10 μM CQ or 20 μM HCQ for 72 h and their effects on cell proliferation and differentiation were determined. The specific soluble guanylyl cyclase inhibitor LY83583, which acts by inhibiting the production of cGMP and KT5823, a potent, selective inhibitor of cGMP-dependent protein kinase (PKG) were used in sGC inhibition studies. 5×10^5 cells in duplicates were pretreated with 0.1 μM LY83583 or 1.0 μM of KT5823 for 12 h before adding CQ or HCQ. Equivalent volumes of either medium alone or medium containing DMSO were added to control cultures. The maximum concentration of DMSO in any experiment was 0.1% (v/v), which did not affect Hb F production or cell viability.

2.3. Time-course studies

The K562 cells (1×10^6 cells/ml) were incubated in RPMI 1640 medium supplemented with 10% FBS in the presence of drugs at 37 °C for 72 h. At predetermined time intervals, an aliquot of cells was collected for growth inhibition studies. A parallel control experiment without the addition of drugs was simultaneously performed. The above drug concentrations were used in this assay because preliminary studies showed that these concentrations optimally increased the total hemoglobin level and Hb F production.

2.4. Determination of total hemoglobin and Hb F

Hemoglobin concentration was determined using the method of Wanda et al. [27] with slight modification. In brief, cultured cells were washed three times with PBS and 300 μl of duplicated samples were centrifuged for 2 min at 900 × *g* at ambient temperature. Following the removal of the supernatant, cell pellets were dispersed in 100 μl 2% Igepal CA-630 (Sigma Chemical Co, St. Louis, MO, USA) per duplicate in 0.05 M Tris–0.4 glycine buffer, pH 7.4, and 0.05 ml benzidine reagent was added. After 30 min at ambient temperature, each sample was made up to 1 ml with 0.05 M Tris–glycin buffer, pH 7.4, followed by centrifugation for 5 min at 900 × *g*. Clear supernatant were read at 425 nm. Standards were prepared using crystalline human hemoglobin (Sigma Chemical Co, St. Louis, MO). Protein concentration of the lysate was determined by the method of Lowry and the amount of total hemoglobin was expressed as μg/(ml mg protein) or μg/(ml 10^6 cells).

Concentration of Hb F was measured by ELISA based on a two-antibody sandwich principle as described elsewhere [28] with slight modification. Briefly, microtiter-plates were coated at 37 °C for 1 h with 100 μl sheep anti-Hb F-antibody (Bethyl Laboratories, Montgomery TX, USA) diluted 1:100 in 100 mM Na_2CO_3/$NaHCO_3$, pH 9.6. After washing four times with Tris-buffered-saline containing 0.02% (v/v) Tween-20 (TBS-T), unspecific binding sites were blocked with 200 μl 40 mM Tris/HCl pH 7.6, containing 137 mM NaCl, 0.02% (v/v) Tween-20, and 3% (w/v) bovine serum albumin (BSA) at 4 °C for 12 h. Plates were further washed four times with TBS-T, followed by addition of 100 μl K562 cell lysate, diluted 1:1000 with diluent-buffer (50 mM Tris/HCl pH 8.0, 140 mM NaCl, 0.05% (v/v) Tween-20, 1.0% (w/v) BSA) to each well and incubated at room temperature for 1 h. After washing four times with TBS-T, 100 μl mouse–anti-human-hemoglobin-gamma-chain IgG_1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:1000 with diluent-buffer, was added and incubated at room temperature for 1 h. Thereafter, wells were washed four times with TBS-T, followed by incubation with 100 μl goat anti-mouse IgG_1-HRP conjugate (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:10,000 with diluent-buffer, at room temperature for 1 h. The wells were washed four times with TBS-T, and bound antibody was detected by addition of 100 μl TMB substrate solution (Bethyl). The reaction was stopped by addition of 100 μl 2N H_2SO_4 and color reaction determined at 450 nm in an ELISA-reader. Fetal hemoglobin standard (Bethyl) was linear ranging from 0 to 400 ng/ml.

2.5. Determination of arginase activity

One million cells were cultured in the absence or presence of studied agents for 72 h. Thereafter, cells were washed twice with PBS followed by addition of 100 μl of lysis buffer (30 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 10% glycerol) and were centrifuged to remove cell debris. Arginase activity was assayed from lysed samples using a modified spectrophotometric assay that was originally developed by Chinard [29,30].

Briefly, 16.67 μl of cell lysate was added to a reaction buffer (100 mM Tris–HCl buffer) at pH, 7.4 containing 10 mM $MnCl_2$. Arginine was added to the sample to initiate the reaction, followed by incubation at 37 °C in a shaking water bath for 20 min. The reaction was stopped by the addition of 55.6 μl of 0.72 M HCl. Further, 0.22 ml of 6% ninhydrin dissolved in 2-methoxyethanol was added and the sample was boiled at 100 °C for 25 min. Thereafter, the sample reaction mixture was allowed to cool to room temperature and the absorbance was measured at 505 nm using the PowerWave XS Spectrophotometer (Bio-Tek, Winooski, VT, USA). The level of arginase activity was expressed as μmoles of ornithine produced per min (U) per milligram protein. Inhibition of arginase activity was monitored by measuring rates of ornithine produced in the presence of CQ or HCQ at defined concentrations.

2.6. Determination of arginase-1 levels in K562 cells

One million cells were grown in the medium containing different concentrations of CQ or HCQ for 72 h and the levels of human arginase-1 was determined in the lysed cell hemolysate by immunosorbent assay (Cell Sciences Inc., Canton, MA, USA) according to the manufacturer's protocol.

2.7. Determination of cGMP levels in K562 cells

One million cells were grown for different periods of time in the medium containing CQ (10 μM) or HCQ (20 μM). 3-Isobutyl-1-methylxanthine (phosphodiesterase inhibitor) was added to the culture medium 30 min prior to harvesting the cells at a final concentration of 0.5 mM. Intracellular cGMP levels were measured by using the cGMP enzyme-immunoassay system (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) according to the manufacturer's protocol.

2.8. Statistical analyses

Data are expressed as mean ± standard error of the mean (S.E.M.). The Student's *t*-test was used for comparison of the arginase activities or Hb F levels between studied pairs. For multiple comparisons, the significance of differences was evaluated by the modified *t*-test according to Dunnett by use of the computer program, Graphpad Prism Instat (GraphPad Software, San Diego, CA, USA). The level of significance was set at $P < 0.05$.

3. Results

3.1. Effects of CQ or HCQ on growth and differentiation of K562 cells

The human erythroleukemic cells were incubated with various concentrations of CQ or HCQ for 72 h and the effects of these drugs on growth was determined. The IC_{50} (drug concentration at which 50% of cells were inhibited) values for CQ and HCQ were 8 and 17 μM, respectively (Fig. 1A). At concentrations >50 μM, CQ showed more significant cytotoxicity than HCQ and the growth inhibitory and cytotoxic effects of both compounds were concentration-dependent. To ascertain whether CQ or HCQ induces K562 cells toward erythroid differentiation, cells were treated with different concentrations of CQ or HCQ for 72 h and total hemoglobin levels (marker of erythroid differentiation) were examined. As

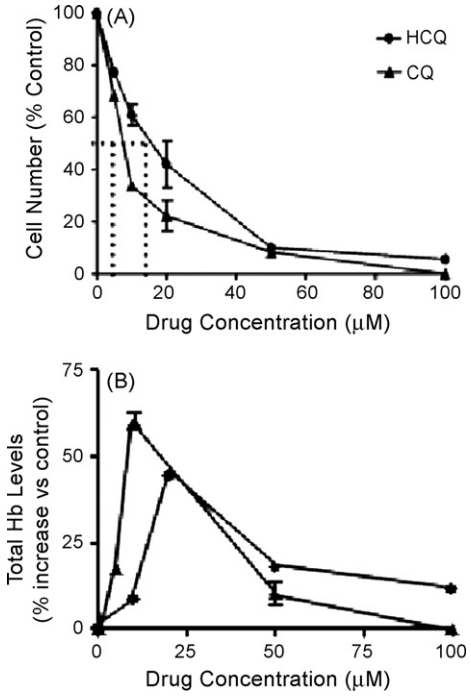

Fig. 1 – Dose–response study on the effects of CQ or HCQ on growth and differentiation of K562 cells. (A) Cells were cultured and treated with different concentrations of CQ or HCQ for 72 h. Cell viability and growth inhibition were determined by Trypan blue exclusion assay. The vertical broken lines indicate the IC_{50} concentration of each individual drug. The degree of cell differentiation (B) was determined by estimating the total hemoglobin levels in a 72 h culture in the presence or absence of the drugs. Results were expressed as percent increase of the respective control values. The mean ± S.E.M. of duplicate samples of four separate assays is shown.

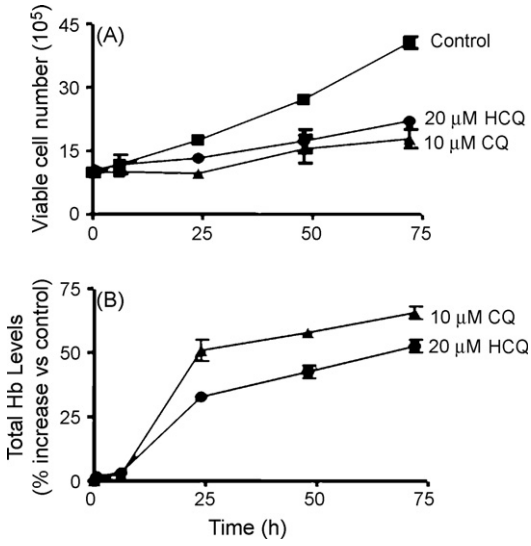

Fig. 2 – Time-course study on the effects of CQ or HCQ on growth and differentiation of K562 cells. Cells were cultured with CQ or HCQ for 72 h. At time intervals, an aliquot of cells was collected to determine the extent of cell inhibition (A), by using the Trypan blue exclusion assay and (B), the extent of erythroid differentiation of K562 cells was assessed by hemoglobinization as detailed in Section 2. The means ± S.E.M. of duplicate assays are given and plotted as the total viable cell number or percent increase of total hemoglobin vs. their respective control values.

clearly evident, both compounds induced erythroid differentiation, although CQ (10 μM) appears to be a more potent inducer of hemoglobinization than HCQ (20 μM) at their respective maximum efficacious doses. However, above their maximum efficacious doses, the amount of accumulated total hemoglobin levels decreased considerably (Fig. 1B). Fig. 2A and B show the time-course of drug-induced inhibition of cell proliferation and differentiation. As shown, CQ or HCQ maximally induced K562 cells towards erythroid differentiation at 72 h. It is important to note that at concentrations of <10 μM CQ or <20 μM HCQ, the effects on the inhibition of arginase activity or induction of cell differentiation were minimal (results not shown).

3.2. *Involvement of arginase modulation by CQ or HCQ on Hb F program in K562 cells*

Recently, it has been shown that activation of guanylate cyclase activity secondary to NO regulation by appropriate agents induced Hb F production [10,11]. In SCD however, increased arginase activity scavenges endothelium derived NO secondary to reduced arginine availability. We then sought to determine the effect of CQ or HCQ-induced modulation of arginase activity on erythroid program. As showed (Figs. 3–5), there was approximately a 1.3- to 3.4-fold increase in the levels of Hb F in cells treated with 10 μM CQ or 20 μM HCQ compared to untreated controls. Also, there was a direct correlation between the percentage of F-cells (as assessed by F-cells staining kit; Sigma Chemical Co, St. Louis, MO), and the levels of Hb F in drug-treated cells (result not shown). In the next experiment, we assessed the role of the arginine–polyamine pathway in the induction of differentiation of K562 cells by CQ or HCQ. Cells were treated with 10 μM CQ or 20 μM HCQ in the presence or absence of spermine (5 μM) or spermidine (5 μM) for 72 h. Our results indicate that spermine and spermidine (intermediate products of the polyamine pathway) alone moderately inhibited cell proliferation and reduced arginase activity. This effect of spermine (not spermidine) resulted in moderate cell differentiation. Surprisingly, upon co-incubation of cells with CQ, spermine or spermidine completely reverted the CQ-mediated inhibitory effects on arginase activity. However, this increased arginase activity failed to inhibit the commitment of cell towards erythroid program induced by CQ (Fig. 3A and B), thus indicating that CQ-induced erythroid differentiation probably precedes arginase modulation. As shown in Fig. 4A, co-incubation of cells with the polyamines and HCQ also resulted in the stimulation of arginase activity. However the HCQ-induced production of Hb F in the presence of spermidine (not spermine) was severely compromised (Fig. 4B), suggesting the involvement of different molecular interaction(s) of CQ and HCQ with spermine or spermidine in the induction of K562 cells towards erythroid maturation. It is also possible that CQ or HCQ modulate the

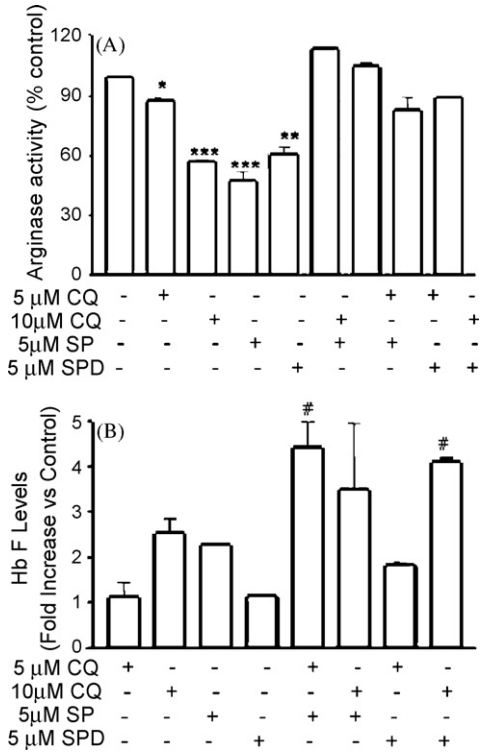

Fig. 3 – Combined effects of CQ and spermine or spermidine on arginase activity and Hb F production in K562 cells. Cells were cultured and treated with 5 or 10 μM CQ in the presence or absence of spermine (5 μM) or spermidine (5 μM) for 72 h and the arginase activity or Hb F production were determined as described in Section 2. Results were expressed as percentage of the control values (A) or as fold increased of the control values (B). The mean ± S.E.M. of duplicate samples of three separate assays is shown. ***P < 0.001; **P < 0.01 compared with control samples. #P < 0.05 compared with the corresponding CQ-treated samples alone.

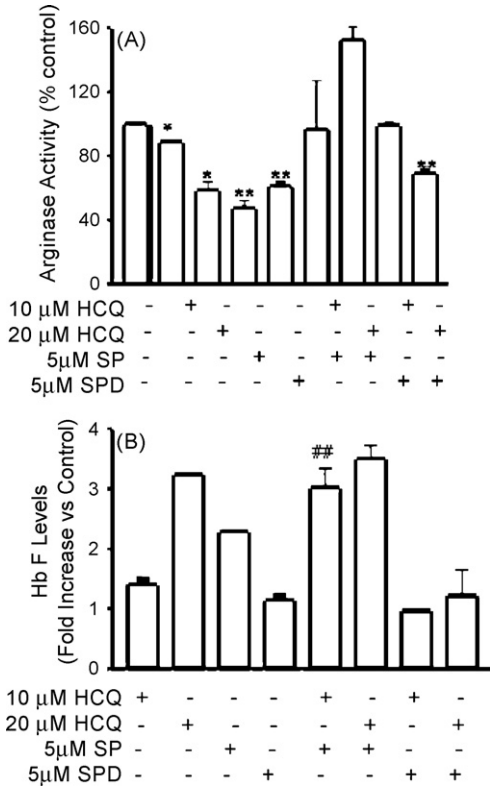

Fig. 4 – Combined effects of HCQ and spermine or spermidine on arginase activity and Hb F production in K562 cells. Cells were cultured and treated with 10 or 20 μM HCQ in the presence or absence of spermine (5 μM) or spermidine (5 μM) for 72 h and the arginase activity or Hb F production were determined as described in Section 2. Results were expressed as percentage of the control values (A) or as fold increased of the control values (B). The mean ± S.E.M. of duplicate samples of three separate assays is shown. **P < 0.01; *P < 0.05 compared with control samples. ##P < 0.01 compared with 10 μM HCQ-treated samples alone.

intermediate products of the polyamine pathway, resulting in the induction of K562 cells towards erythroid differentiation.

Since LPS and Br-cAMP are known inducers of arginase activity [31,32], we further investigated the effects of Br-cAMP on arginase activity in lipopolysaccharide (LPS)-stimulated K562 cells. In this study we also observed the stimulatory effects of LPS and Br-cAMP on arginase activity. However, the inhibitory affects of CQ or HCQ on arginase activity could not be reversed by LPS or Br-cAMP (Fig. 5A). Indeed, the addition of LPS or Br-cAMP to CQ or HCQ-treated cells resulted in a significant induction of Hb F production (Fig. 5B). This surprising observation could be explained on the basis of CQ or HCQ-induced inhibition of arginase activity, even in the presence of LPS or Br-cAMP, further supporting the possible involvement of the arginase pathway in the stimulation of Hb F in K562 cells.

3.3. *Effects of CQ or HCQ on human arginase-1 in K562 cells*

Human arginase 1 (A1), which is expressed in hepatic and various extrahepatic tissues, as well as in red blood cells (RBCs), plays a role in regulating arginine levels. We performed experiments using specific anti-human arginase 1 antibody to determine the effects of CQ or HCQ on A1 levels. Cells were treated in the presence or absence of CQ or HCQ for 72 h and the levels of A1 were determined. Our results showed that 10 μM CQ or 20 μM HCQ moderately inhibited A1 in comparison with the control samples. As expected, at higher concentrations both agents significantly inhibited A1 levels (Fig. 6). Overall, the effect of CQ or HCQ on arginase activity was more apparent than their effects on A1 levels (see Figs. 5, 3A and 4A).

3.4. *Mechanism of CQ or HCQ-induced production of Hb F in K562 cells*

Having established the involvement of arginase pathway in CQ or HCQ-induced production of Hb F, we then reasoned that the alteration of arginase activity mediated by CQ or HCQ might affect cGMP levels. To this end, we examined the effects of these drugs on intracellular cGMP levels, since it has been shown that

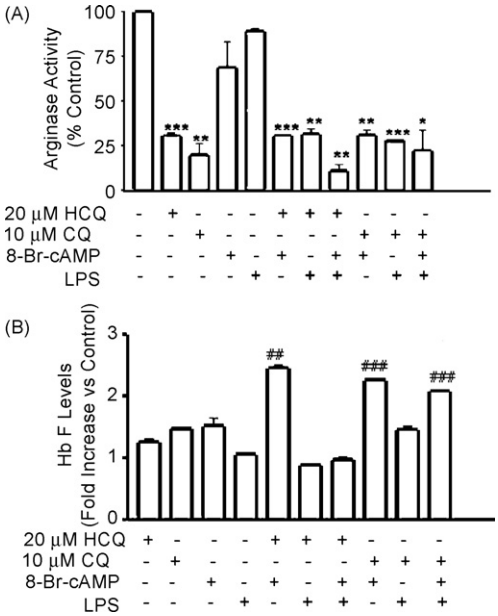

Fig. 5 – Combined effects of CQ or HCQ and LPS or Br-cAMP on arginase activity and Hb F production in K562 cells. Cells were cultured and treated with 10 μM CQ or 20 μM HCQ in the presence or absence of LPS (10 μg/ml) or Br-cAMP (2 μM) for 72 h and the arginase activity expressed as percentage of the control values (A) or the production of Hb F expressed as fold increased of the control values (B) were determined as described in Section 2. The mean ± S.E.M. of duplicate assays of two separate assays is shown. ***P < 0.001; **P < 0.01; *P < 0.05 compared with control samples. ###P < 0.001; ##P < 0.01 compared with the corresponding CQ or HCQ-treated samples alone.

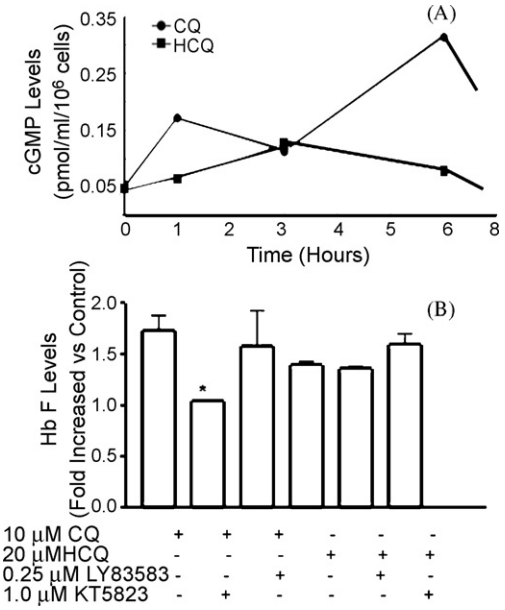

Fig. 7 – Mechanism of CQ- or HCQ-induced production of Hb F in K562 cells. (A) Cultured cells were treated with 10 μM CQ or 20 μM HCQ at predetermined time points, followed by cell treatment with 0.5 mM of 3-isobutyl-1-methylxanthine (phosphodiesterase inhibitor) 30 min prior to harvesting the cells. The intracellular cGMP levels were measured by using the cGMP enzyme-immunoassay system as indicated in Section 2, and the effects of LY83583 and KT5823 (inhibitors of sGC-PKG signaling pathway) on Hb F production in a 72 h cultured cells treated with CQ or HCQ were also determined (B). The mean ± S.E.M. of duplicate assays is shown and plotted as fold increase of the control values. *P < 0.05 compared with CQ-treated samples alone.

some of the most potent Hb F-inducing differentiating agents, such as HU partly utilize the sGC-PKG signaling pathway secondary to NO stimulation [10,11]. K562 cells were cultured in the presence of 10 μM CQ or 20 μM HCQ for 72 h and the levels of

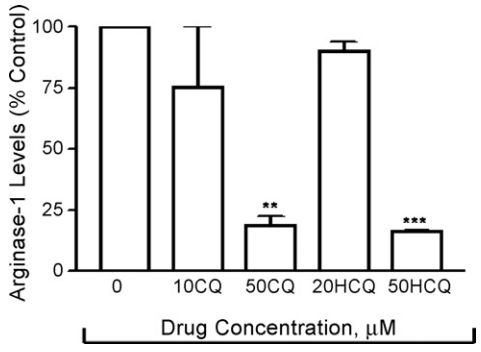

Fig. 6 – Effects of CQ or HCQ on arginase 1 levels in K562 cells. Cells were cultured and treated with different concentrations of CQ or HCQ for 72 h and the levels of arginase 1 was determined by enzyme immunosorbent assay as described in Section 2. The mean ± S.E.M. of duplicate assays of two separate assays is shown and plotted as percentage of the control values. ***P < 0.001; **P < 0.01 compared with control samples.

cGMP were estimated by immunosorbent assay. As shown in Fig. 7A, CQ and HCQ maximally stimulated the intracellular cGMP levels by 6.6- and 3.0-fold at 6 and 3 h respectively. To evaluate these results further, we sought to suppress sGC activity in K562 cells by pretreating cells for 12 h with the sGC inhibitor LY83583 or PKG specific inhibitor KT 5823 before adding CQ or HCQ. As shown in Fig. 7B, there was a significant reduction of Hb F production in CQ-treated cells by KT5823 in comparison with the control samples and the effects of LY83583 on CQ-mediated Hb F production were moderate. On the other hand, we did not observe any significant effects from addition of KT5823 or LY83583 on HCQ-mediated Hb F production. These results indicate that the CQ or HCQ-induced commitment of K562 cells towards erythroid program resulting in Hb F production may partly involve not only the modulation of the arginase pathway but also the participation of other but yet unidentified signaling pathways, including (in the case of CQ) the sGC-PKG signaling pathway.

4. Discussions

We have shown that CQ or HCQ, anti-malarial and anti-rheumatoid drugs, inhibited arginase stimulation leading to

induced differentiation of human erythroleukemic cell line. This enhancement of cells towards erythroid maturation resulted in the potentiation of both total and fetal hemoglobins. The CQ or HCQ dose used for this study was established after a dose–response study and 10 μM CQ or 20 μM HCQ were found to be optimum for the inhibition of arginase activity and enhancement of cell differentiation. In earlier reports, CQ doses from 10 to 200 μM were used in *in vitro* study on the CQ-induced inhibition of proinflammatory cytokines in human erythrocytes [21], which are higher than the doses we employed for K562 cells. We have also documented in this study that the inhibitory effects of CQ or HCQ on arginase activity and cell proliferation could not be reverse by co-incubation of the cell cultures with LPS or Br-cAMP (known stimulators of arginase activity), and this resulted in concomitant enhancement of K562 cells towards erythroid program as assessed by Hb F production. Thus, our results suggest that CQ or HCQ inhibit K562 cell proliferation by inhibiting arginase activity and inducing erythroid differentiation.

Studies have shown that at appropriate concentrations, spermine and spermidine (intermediate products of polyamine pathway) modulate arginase activity leading to altered cell growth [33,34]. In our present study, we have also shown that co-incubation of K562 cells with these polyamines reverted the inhibition of arginase activity as well as the anti-proliferation effects of CQ or HCQ. But the ability of these cells to differentiate in the presence of spermidine (not spermine) and HCQ were significantly compromised in comparison with HCQ-treated cells alone. Hence, it is plausible that the CQ or HCQ-induced effects on K562 cells towards hemoglobin synthesis probably precede their effects on arginase modulation and that there may be different mechanism(s) involved in the molecular interactions between CQ or HCQ and these polyamines. We have also provided evidence that CQ or HCQ-mediated production of Hb F may involve the stimulation of intracellular cGMP levels, with the possible involvement of the sGC-PKG pathway in the case of CQ-mediated effect of K562 cells towards erythroid maturation. In this study, we have not excluded the possibility that other pathways may also play a role in the CQ or HCQ-mediated enhancement K562 cells towards erythroid Hb F program, such as the p38/MAP kinase signaling pathway [35].

Recent experimental and clinical evidence indicate that increased erythrocyte arginase activity may be implicated in several pathological conditions in SCD and other inflammatory disorders [36–39]. Also it has been hypothesized that a dysregulation of the nitric oxide (NO) synthase-arginase pathway secondary to increased arginase activity may contribute to the pathology of NO-disorders such as pulmonary hypertension [38]. However, by modulating the arginase activity, it is feasible that more endogenous substrate (arginine) will be made available for NO production by NO synthase. Increased bioavailability of NO may then induce Hb F production via the sGC-PKG pathway [10,11], thereby ameliorating the clinical course of SCD. Recently we demonstrated that CQ, an immunomodulating drug, inhibits arginase activity in sickle erythrocytes via a competitive mechanism [24]. However to our knowledge information on the CQ or HCQ-induced enhancement of K562 cells towards erythroid maturation resulting in the formation of both total and fetal hemoglobins secondary to the modulation of arginase pathway is unknown.

Although further studies will be necessary to elucidate the mechanism of CQ or HCQ-induced modulation of arginase pathway leading to induced cell commitment towards erythroid program in human progenitor cells as well as in a transgenic mouse model, we have demonstrated, for the first time, the link between the modulation of the polyamine pathway and induced cell accumulation of total and fetal hemoglobin in human erythroleukemic cells when treated with CQ or HCQ.

In conclusion, we believe that our results represent a new and important improvement in understanding the pleiotropic effects of CQ and its derivative HCQ on arginase activity, with specific reference to their participation in the enhancement of Hb F production. Hence, incorporating this template into structure-based enzyme design studies could lead to the production of new forms of efficient arginase inhibitors, which could prove useful as therapeutic regimen in hemoglobinopathies and other related inflammation-mediated diseases.

Acknowledgements

We wish to thank Dr. Steve Leeder for critically reading the manuscript. This work was partially supported by grants KO1 HL076695-01 from the National Institutes of Health.

REFERENCES

[1] Bianchi N, Ongaro F, Chiarabelli C, Gualandi L, Mischiati C, Bergamini P, et al. Induction of erythroid differentiation of human K562 cells by cisplatin analogs. Biochem Pharmacol 2000;60:31–40.
[2] Bianchi N, Osti F, Rutigliano C, Ginanni Corradini F, Borsetti E, Tomassetti M, et al. The DNA-binding drugs mithramycin and chromomycin are powerful inducers of erythroid differentiation of human K562 cells. Brit J Haematol 1999;104:258–65.
[3] Charache S, Dover GJ, Moore RD, Eckert S, Ballas SK, Koshy M, et al. Hydroxyurea: effects on hemoglobin F production in patients with sickle cell anemia. Blood 1992;79:2555–65.
[4] CioeÁ L, McNab Á, Hubbell A, Meo HR, Curtis P, Rovera G. Differential expression of the globin genes in human leukemia K562 (S) cells induced to differentiate by haemin or butyric acid. Cancer Res 1981;41:237–43.
[5] Dover GJ, Brusilow S, Samid D. Increased fetal haemoglobin in patients receiving sodium 4-phenylbutyrate. New Engl J Med 1992;327:569–70.
[6] Fibach E, Burke LP, Schechter AN, Noguchi CT, Rodgers GP. Hydroxyurea increases fetal hemoglobin in cultured erythroid cells derived from normal individuals and patients with sickle cell anemia or beta-thalassemia. Blood 1993;81:1630–5.
[7] Ley TJ, DeSimone J, Anagnou NP, Keller GH, Humphries RK, Turner PH, et al. 5-Azacytidine selectively increases gamma-globin synthesis in a patient with beta1 thalassaemia. New Engl J Med 1982;307:1469–75.
[8] Platt OS, Brambilla DJ, Rosse WF, Milner PF, Castro O, Steinberg MH, et al. Mortality in sickle cell disease. Life expectancy and risk factors for early death. N Engl J Med 1994;330:1639–44.

[9] Mozzarelli A, Eaton WA, Hofrichter J. Delay time of hemoglobin S gelation prevents most cells from sickling in vivo. Science 1987;237:500–6.
[10] Cokic VP, Smith RD, Beleslin-Cokic BB, Njoroge JM, Miller JL, Gladwin MT, et al. Hydroxyurea induces fetal hemoglobin by the nitric oxide-dependent activation of soluble guanylyl cyclase. J Clin Invest 2003;111:231–9.
[11] Ikuta T, Ausenda S, Cappellini MD. Mechanism for fetal globin gene expression: role of the soluble guanylate cyclase–cGMP-dependent protein kinase pathway. Proc Natl Acad Sci USA 2001;98:1847–52.
[12] Azizi E, Dror Y, Wallis K. Arginase activity in erythrocytes of healthy and ill children. Clin Chim Acta 1970;28: 391–6.
[13] Kim PS, Iyer RK, Lu KV, Yu H, Karimi A, Kern RM, et al. Expression of the liver form of arginase in erythrocytes. Mol Genet Metab 2002;76:100–10.
[14] Munder M, Eichmann K, Morán JM, Centeno F, Soler G, Modolell M. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. J Immunol 1999;163:3771–7.
[15] Zea AH, Rodriguez PC, Atkins MB, Hernandez C, Signoretti S, Zabaleta J, et al. Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. Cancer Res 2005;65:3044–8.
[16] Hillery CA, Panepinto JA. Pathophysiology of stroke in sickle cell disease. Microcirculation 2004;11:195–208.
[17] Kepka-lenhart D, Mistry SK, Wu G, Sidney M, Morris SMJR. Arginase I: a limiting factor for nitric oxide and polyamine synthesis by activated macrophages? Am J Physiol Reg Integr Comp Physiol 2000;279:R2237–42.
[18] Satriano J. Arginine pathways and the inflammatory response: interregulation of nitric oxide and polyamines: review article. Amino Acids 2004;26:321–9.
[19] Wallace DJ. Antimalarial agents and lupus. Rheum Dis Clin North Am 1994;20:243–63.
[20] Ertel W, Morrison MH, Ayala A, Chaudry IH. Chloroquine attenuates hemorrhagic shock-induced suppression of Kupffer cell antigen presentation and major histocompatibility complex class II antigen expression through blockade of tumor necrosis factor and prostaglandin release. Blood 1991;78:1781–8.
[21] Karres I, Kremer JP, Dietl I, Steckholzer U, Jochum M, Ertel W. Chloroquine inhibits proinflammatory cytokine release into human whole blood. Am J Physiol Reg Integr Comp Physiol 1998;274:1058–64.
[22] Salmeron G, Lipsky PE. Immunosuppressive potential of antimalarials. Am J Med 1983;75:19–24.
[23] Weber SM, Levitz SM. Chloroquine interferes with lipopolysaccharide-induced TNF-alpha gene expression by a nonlysosomotropic mechanism. J Immunol 2000;165:1534–40.
[24] Iyamu EW, Ekekezie C, Woods GM. In vitro evidence of the inhibitory capacity of chloroquine on arginase activity in sickle erythrocytes. Brit J Haematol 2007;139:337–43.
[25] Iyamu EW, Cecil R, Parkin L, Woods G, Ohene-Frempong K, Asakura T. Modulation of erythrocyte arginase activity in sickle cell disease patients during hydroxyurea therapy. Brit J Haematol 2005;131:389–94.
[26] Chou T, Talalay P. Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 1989;22:27–55.
[27] Wanda PE, Lee LT, Howe C. A spectroscopic method for measuring hemoglobin in erythroleukemic (K562) cells. J Histochem Cytochem 1981;29:1441–4.
[28] Witt O, Moenkemeyer S, Roenndahl G, Erdlenbruch B, Reinhardt D, Kanbach K, et al. Induction of fetal hemoglobin expression by the histone-deacetylase inhibitor apicidin. Blood 2003;101:2001–7.
[29] Chinard FP. Photometric estimation of proline and ornithine. J Biol Chem 1952;199:91–5.
[30] Tormanen CD. Substrate inhibition of rat and kidney arginase with fluoride. J Inorg Biochem 1997;66:111–8.
[31] Ryan JL, Yohe WB, Morrison DC. Stimulation of peritoneal cell arginase by bacterial lipopolysaccharides. Am J Pathol 1980;99:451–61.
[32] Wei LH, Jacobs AT, Morris JR SM, Ignarro LJ. IL-4 and IL-13 upregulate arginase I expression by cAMP and JAK/STAT6 pathways in vascular smooth muscle cells. Am J Physiol Cell Physiol 2000;279:C248–56.
[33] Jänne J, Pösö H, Raina A. Polyamines in rapid growth and cancer. Biochim Biophys Acta 1978;473:241–93.
[34] Rupniak HT, Paul D. Inhibition of spermidine and spermine synthesis leads to growth arrest of rat embryo fibroblasts in G1. J Cell Physiol 1978;94:161–70.
[35] Pace BS, Qian XH, Sangerman J, Ofori-Acquah SF, Baliga BS, Han J. Critz SD. p38 MAP kinase activation mediates gamma-globin gene induction in erythroid progenitors. Exp Hematol 2003;31:1089–96.
[36] Iniesta V, Carcelén J, Molano I, Peixoto PMV, Redondo E, Parra P, et al. I Induction during *Leishmania major* infection mediates the development of disease. Infect Immun 2005;73:6085–90.
[37] Meurs H, Maarsingh H, Zaagsma J. Arginase and asthma: novel insights into nitric oxide homeostasis and airway hyperresponsiveness. Trends Pharmacol Sci 2003;24:450–5.
[38] Morris CR, Kato GJ, Poljakovic M, Wang X, Blackwelder WC, Sachdev V, et al. Dysregulated arginine metabolism, hemolysis-associated pulmonary hypertension, and mortality in sickle cell disease. JAMA 2005;294:81–90.
[39] Morris CR, Poljakovic M, Lavrisha L, Machado L, Kuypers FA, Morris Jr SM. Decreased arginine bioavailability and increased serum arginase activity in asthma. Am J Resp Crit Care Med 2004;170:148–53.