Biologic Functions of Hydroxychloroquine in Disease

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Chloroquine (CQ) and Hydroxychloroquine (HCQ), initially utilized in the treatment of malaria, have developed a long list of applications. Despite their clinical relevance, their mechanisms of action are not clearly defined. Major pathways by which these agents are proposed to function include alkalinization of lysosomes and endosomes, downregulation of C-X-C chemokine receptor type 4 (CXCR4) expression, high-mobility group box 1 protein (HMGB1) inhibition, alteration of intracellular calcium, and prevention of thrombus formation.

Hydroxychloroquine Chloroquine malaria cancer

1. Introduction

Chloroquine (CQ) and its derivative, hydroxychloroquine (HCQ), are well-known, multi-use drugs with applications in anti-malarial and anti-viral treatment, autoimmune diseases, and neoplastic processes [1][2][3][4]. While CQ and HCQ have been used for decades, new uses continue to be discovered. However, despite their widespread use, the mechanism of action is poorly understood. In fact, numerous mechanisms with overlapping pathways have been proposed.

2. Biochemistry and Pharmacology

Chloroquine and hydroxychloroquine are 4-aminoquinolines with anti-malarial, anti-viral, anti-inflammatory, and anti-cancer applications [5]. Structurally, they differ by only one hydroxyl group. Though CQ has been in use for the better part of a century, its pharmacokinetics were not studied in detail until approximately the 1980s. Using liquid chromatographic techniques with diethyl ether extraction to identify CQ and desethylchloroquine (main metabolite), Gustafsson et al. studied chloroquine concentrations in healthy individuals after single dose administration with either intravenous (IV) or per os (PO) formulation. They found the drug could be detected in urine samples 23–52 days after administration with massive volumes of distribution (Vd) ranging from 111–285 L/kg [6]. Large Vd were further validated by Frisk-Holmberg et al. who showed up to 800 L/kg when calculated by plasma concentrations; however, whole blood concentrations were 8–10 times higher and, consequently, had Vd approximately 10 times lower [7]. This effect is explained by the finding that chloroquine is concentrated in erythrocytes and is approximately 2–5 times higher in red blood cells than in plasma [6]. Further explanation of such large Vd is likely due to the basic nature of chloroquine and its affinity for lysosomal uptake [8][9]. Bioavailability of solution and pill form has been reported ranging from 78% to nearly 100%, with the higher value coming from later studies. The

high bioavailability is attributed to rapid distribution into erythrocytes and thus low plasma levels exposed to first pass hepatic metabolism [6][10]. Similar findings were observed in studies of chloroquine malaria treatment in children [11]. Pregnancy has been shown to decrease half-lives and Vd [12]. The limited amount of chloroquine and desethylchloroquine in plasma is bound to albumin [13][14]. Furthermore, plasma levels varied by enantiomer with (S)-chloroquine plasma levels being approximately 66% bound and (R)-chloroquine 42% bound [15]. Unmetabolized CQ is excreted primarily in urine [16]. A detailed reviewed of chloroquine pharmacokinetics was previously completed by White and more recently by Ducharme and Farinotti [16][17].

3. Alkalinization of Lysosomes and Endosomes

CQ and HCQ's basic properties allow for the drugs to accumulate in, and alkalinize, the acidic environments of lysosomes and endosomes. Each of these organelles contribute to the processes of cell death and cell signaling. Endosomes play an important role in the entry and replication of several viruses, thrombus formation in autoimmune disorders, and cell signaling through endocytic Toll-like receptors (TLRs). Lysosomes, on the other hand, drive autophagy and cell death.

Autophagy is an important cellular process where catabolism of cellular components occurs in the settings of nutrient deprivation, hypoxia, and other cell stressors including chemotherapy and radiation [18]. This process can serve multiple purposes, such as energy production in hypoxic and nutrient-deprived environments, and clearance of damaged organelles and reactive oxygen species (ROS) [19].

Based on the initial stressor signal, different pathways are involved in activating autophagy. The common pathway converges on the formation of the autophagosome, a double membrane structure that encloses cellular components. The autophagosome then fuses with a lysosome, allowing for degradation of its contents via lysosome hydrolases [19][20]. CQ/HCQs alkalinization of lysosomes prevents this fusion, as well as impairs the function of lysosomal hydrolases, resulting in autophagy inhibition and impaired lysosome hydrolase function [21]. CQ's known risk of ocular toxicity has been attributed to this dysfunction of lysosome hydrolases in retinal pigment epithelium (RPE). Lysosomal dysfunction has been shown to lead to an accumulation of lipofuscin, which can lead to retinal toxicity [22][23].

The consequences of autophagy inhibition are numerous. Autophagy has been implicated in carcinogenesis, disease progression, and even metastasis [21][24][25][26]. Tumors with high proliferation rates often outgrow their blood supply and thus, their source of nutrients. Autophagy can serve as a source of fuel in these settings, and therefore, it is no surprise increased rates of autophagy are found in many cancers [21][24][25][26][27]. Pancreatic cancer, for example, has a strong link to increased autophagy and tumor grade, resulting in poor prognosis [24][28]. Yang et al. demonstrated inhibition of autophagy, through genetic means or use of CQ, led to accumulation of ROS which induced DNA damage and decreased cancer cell growth in vitro. Furthermore, inhibition of autophagy with CQ resulted in tumor regression and prolonged survival in mouse models [29]. Likewise, cancer stem cells, which play an important role in tumor initiation, metastasis, recurrence, and chemoresistance, utilize autophagy, with CQ treatment leading to regression and improved outcomes [30][31].

Autophagy may also mediate resistance to chemotherapy. Upregulation of autophagy is seen following multiple chemotherapy agents [32]. To clarify the role of autophagy in chemotherapy resistance, genetic silencing of autophagy-related genes (ATGs) has been tested in the setting of chemoresistant cancer cell lines. Genetic silencing was shown to sensitize previously chemoresistant cells to therapy [33]. Hashimoto et al. demonstrated an increase in autophagy following treatment of pancreatic cancer cells with either 5-fluorouracil (5-FU) or gemcitabine. However, treatment in combination with chloroquine reduced autophagy and potentiated the antiproliferative effects of 5-FU and gemcitabine [34]. Glioblastoma cells were also found to utilize higher rates of autophagy to overcome treatment with Bevacizumab, a monoclonal antibody to vascular endothelial growth factor (VEGF). Chloroquine and hydroxychloroquine reverse this resistance in multiple studies [35][36]. These studies indicate that although CQ/HCQ alone have shown antitumor effects, they may be best utilized as a combination therapy.

In addition to the effect on oncologic cells from autophagy, it also appears to play an important role in activating cancer supporting cells. Pancreatic stellate cells (PSCs) protect the tumor from the immune system's antitumor defense by creating a strong, fibrous stroma around the tumor which decreases T cell infiltration. Endo et al. linked autophagy with PSC activation and, as with pancreatic cancer cells, associated this increased rate of autophagy to a poor prognosis. By inhibiting autophagy with chloroquine, the authors were able to demonstrate conversion of PSCs to a quiescent state as well as a decrease in extracellular matrix accumulation and tumor volumes [37]. Similarly, cancer associated fibroblasts (CAFs) in breast cancer enhance the growth and metastatic potential of breast tumors. Caveolin-1 (Cav-1) is a structural protein and transformation suppressor expressed by healthy fibroblasts. Breast cancer cells are able to downregulate the expression of Cav-1, leading to early disease progression and poor prognosis. Interestingly, CQ was able to restore Cav-1 expression, indicating cancer cells may use autophagy to degrade antitumor structures [27].

Autophagy also plays a role in multiple autoimmune diseases via antigen processing and presentation, T cell activation, and cytokine processing [38][39][40]. Overactivation of T cells results in the body incorrectly targeting self-antigens leading to cell death, extensive inflammation, and organ damage [41]. Through autophagy inhibition, CQ and HCQ prevent autoantigen presentation in antigen presenting cells and B cells, resulting in decreased T cell activation [40]. Rheumatoid arthritis (RA) results in a dysregulation in autophagy and is characterized by synovial inflammation, increased bone catabolism, and damage to cartilage and bone. RA patients develop autoantibodies, often to citrullinated proteins. Fibroblast-like synoviocytes (FLS) are found infiltrating cartilage and bone surfaces and can deposit collagen and α-smooth muscle actin causing synovial fibrosis. High levels of autophagy in RA FLS allow for prolonged survival and correlate with increased levels of antibodies against citrullinated proteins [38].

While lysosomes can support cell survival through autophagy, they can also promote cell death. Cell death can occur through cell necrosis or apoptosis, both of which can be impacted by lysosomes. Lysosomal death is initiated by lysosomal membrane permeabilization (LMP) which allows the translocation of lysosomal enzymes into the cytoplasm instigating cell death [42]. CQ and HCQ are capable of causing permeabilization of not only lysosomal membranes, but also mitochondrial and plasma membranes. Boya et al. showed that HCQ accumulation in lysosomes resulted in increased lysosomal volume followed by lysosomal, mitochondrial, and plasma membrane

permeabilization [43]. Extensive permeabilization, as well as lysosomal hydrolase activity within the cytoplasm, resulted in cell death. LMP induction may be an additional mechanism by which CQ overcomes resistance to chemotherapy. For example, patients with non-small-cell lung cancer (NSCLC) unable to receive immunotherapy often receive chemotherapy. However, resistance forms quickly. CQ was shown to induce LMP leading to apoptosis of NSCLC cells [44]. This effect has also been demonstrated with CQ treatment in conjunction with PI3K/mTOR inhibitors [45][46].

Chloroquine's role in the treatment of malaria has also been attributed to the alkalinization of a type of secondary lysosome called a digestive vesicle (DV) [47]. Malaria, caused by different species of the parasite Plasmodium, is characterized by parasitic invasion of host red blood cells (RBCs). Plasmodium degrades hemoglobin within DVs and utilizes the amino acid products. Heme is also released during this process and is toxic to parasites. However, in the acidic environment of DVs it is quickly converted to the nontoxic hemozoin. This process is inhibited in the setting of CQ induced alkalinization of DVs resulting in heme toxicity to parasites. Plasmodium quickly adapts, though, resulting in widespread CQ resistance. This is secondary to a mutation in the plasmodium falciparum chloroquine resistance transporter gene (pfcrt) which allows for the efflux of CQ out of DV through a transporter protein [48]. Specifically, the transporter takes on a configuration that produces an overall negative charge, attracting and sequestering positively charged compounds such as CQ [49].

Alkalinization of acidic compartments by CQ also impacts endosome function. Endosomes are a critical part of endocytosis, a process which propagates cell signaling and allows them to internalize aspects of the surrounding environment. Viruses commonly utilize endocytosis to gain entry into a cell. CQ has been shown to decrease intracellular viral accumulation of multiple viruses, including Borna virus, HIV, Hepatitis A, Zika virus, Hepatitis C, Dengue virus, and Ebola [50][51][52][53][54][55]. Viral replication is also dependent on organelle pH for intracellular trafficking, unpacking, and post-translational modification [54]. Importantly, other antiviral mechanisms have been proposed separate from the effects of CQ on organelle pH. CQ has been shown to inhibit glycosylation, a necessary process for the glycosylation of viral envelopes and subsequent release [50]. Another possible mechanism is proposed by the inhibition of arachidonic acid metabolism and activation of NFκB, thus decreasing transcription of viral DNA [56].

Some Toll-like receptors (TLRs) depend on endosomal function for the transmission of their signal. TLRs are transmembrane proteins with important functions in innate immunity and inflammation. The proteins are located either on the plasma membrane or endosomal membrane. Endocytic TLRs 3, 7, 8, and 9 require internalization of ligands to stimulate activity, a process which is inhibited by compartment alkalinization by CQ [57][58][59][60][61][62]. In fact, Rutz et al. identified CQs ability to inhibit TLR9 signaling to be pH dependent, supporting this proposed mechanism [63]. Endocytic TLRs are involved in sepsis-induced mortality and acute kidney injury (AKI) in mouse models. Treatment with CQ decreased AKI, TLR protein in the spleen, and systemic inflammation as well as improved the survival rate [64]. Likewise, treatment with CQ prevented bacterial DNA-induced TLR signaling of the inflammatory response to sepsis [65]. TLR inhibition has many downstream effects, including reduced cytokine production, and impaired recognition of immune complexes by endosomal TLRs in autoimmune diseases [66]. Additionally, TLR9 may have a role in the pathogenesis of type I diabetes. CQ treatment decreased development of

diabetes and improved islet cell function. Of note, there is evidence that CQs effect on TLRs may extend beyond pH modifications. Kuznik et al. recently demonstrated CQs effect on TLRs is present even with only minimal changes in endosomal pH. In fact, CQ could directly bind ligands in order to prevent their binding to TLRs. Further revealed was CQ's capability to directly bind TLR ligands, preventing their binding to receptors. CQ was found to inhibit the function of TLRs 3, 7, and 9, but acted as an agonist for TLR8 [67]. Similarly, Zhang et al. demonstrated the reversibility of CQs effect via addition of a TLR9 agonist, again contradicting the theory of alkalinization as the sole source of the mechanism [68].

4. C-X-C Chemokine Receptor Type 4

C-X-C chemokine receptor type 4 (CXCR4) is a chemokine receptor that, along with its ligand C-X-C motif chemokine ligand 12 (CXCL12), impacts many physiologic as well as pathologic processes. The CXCR4/CXCL12 axis is widely expressed throughout the human body, and their downstream effects of receptor binding result in gene transcription, cell proliferation and survival, and cellular adhesion and migration [69][70]. The embryonic vitality of the CXCR4 receptor and chemokine has been demonstrated in murine models; and the physiologic functions of embryogenesis, hematopoiesis, brain development, and leucocyte trafficking towards sites of inflammation have been well established [70][71][72][73][74]. Pathologically, a role in multiple autoimmune diseases, stroke, and the cellular entry of human immunodeficiency virus has also been studied [70][74][75][76]. In oncologic disease, CXCR4 has been found to be frequently overexpressed in malignant cells and linked to primary tumor growth, angiogenesis, tumor invasion of surrounding tissues, and metastasis [69][70][73][74][77][78][79]. Due to the pathologic function of this axis, it has gained attention from researchers searching for a viable inhibitor. Chloroquine-containing products have been found to downregulate CXCR4 expression [69][80][81].

In 2012, Kim et al. observed decreased CXCR4-mediated pancreatic cancer cell signaling and proliferation in vitro [82]. Further in vitro experimentation by Balic et al. in 2014 showed a significant decrease in the number of circulating tumor cells in pancreatic cancer treated with chloroquine. The inhibition was found to reduce phosphorylation of extracellular signal-regulated kinase (ERK) and signal transducer and activator of transcription 3 (STAT3) and showed potential to assist with the control of metastatic disease [83]. Inhibition of CXCR4 with CQ has also been shown to delay tumor progression in esophageal cancer in mice [84]. Through this pathway of inhibition, effects on tumor vasculature and immune system function have also been noted [85]. In 2016, Yu et al. published two studies where synthesized CQ was used to decrease cell surface expression of CXCR4 in oncologic cells and proved to have both antimetastatic properties in addition to causing less toxicity than its parent drug, hydroxychloroquine [86][87]. While this mechanism of action for chloroquine products has not been the most studied, there is evidence supporting its further research and how it may help the treatment of oncologic disease.

5. High-Mobility Group Box 1 Protein

High-mobility group box 1 protein is a DNA-binding protein with both intra- and extracellular functions through many receptors such as that found in advanced glycation end products (RAGE), T cell immunoglobulin domain and

mucin domain-3, and TLR4. HMGB1s downstream effects are abundant and include the following: transcription regulation, autophagy initiation, carcinogenesis, angiogenesis, potentiation of inflammation and ischemia, cytokine production, hypercoagulability, NETosis, and sepsis [58][88][89][90][91][92][93][94][95]. In the presence of ROS, there is an upregulation of RAGE expression, which binds HMGB1 resulting in the activation of multiple pathways. First, TLR9 can be stimulated, resulting in the release of inflammatory cytokines. Second, IL-6 release activates STAT3, a process that can both enhance autophagy and increase CXCR4 expression. Third, the RAGE-HMGB1 complex also directly triggers autophagy. As discussed, CQ can impact multiple aspects of these pathways, including TLR9, CXCR4, and autophagy. However, CQ has also been shown to inhibit release of HMGB1 in septic mice resulting in improved mortality [92]. Furthermore, it prevented release of HMGB1 from monocytes following stimulation with LPS or IFNγ [96]. This impact has not yet been studied extensively in other pathologies but represents an additional potential target of CQ.

6. Alteration of Intracellular Calcium

Intracellular calcium stores are an important part of cell signaling with increased intracellular calcium levels resulting in signal propagation. Platelet aggregation, for instance, often requires alterations in intracellular calcium levels. Platelet aggregation can be induced by multiple stimulants, including phorbol-myristate-acetate (PMA), calcium (Ca) ionophores, and thrombin.

PMA and thrombin act via protein kinase C (PKC), resulting in a mobilization of intracellular calcium stores. On the other hand, Ca ionophores do not require membrane receptors and utilize influx of extracellular calcium. Ca ionophore and thrombin stimulation both induce release of phospholipase A2 (PLA2), leading to arachidonic acid (AA) liberation from plasma membrane phospholipids. The arachidonic acid cascade is critical for platelet aggregation as it yields thromboxane A2 (TXA2) which is important for aggregation and vasoconstriction. CQ is capable of inhibiting platelet aggregation secondary to PMA, Ca ionophore, and thrombin stimulation; however, it is less potent in relation to Ca ionophore stimulation. This difference may indicate that CQ plays more prominence on intracellular calcium than extracellular [97][98]. Research has shown CQ can also inhibit the arachidonic acid pathway via inhibition of PLA2, leading to reduction in TXA2 production [98][99].

PAD4 function has also been shown to be dependent on high intracellular calcium levels. PAD4 is an enzyme that citrullinates DNA histones resulting in decondensed chromatin. Its actions are essential to NETosis as PAD4 deficient mouse neutrophils are unable to form NETs [100]. PAD4 inhibitors also reduce NET formation in mouse and human neutrophils [101]. As discussed, NETs play an important role in multiple stages of cancer, autoimmune disease, and thrombus formation. PAD4 function disruption via alteration of intracellular calcium stores may be yet another mechanism by which HCQ inhibits NET formation.

7. Prevention of Thrombosis

Autoimmune diseases such as antiphospholipid syndrome (APS) and systemic lupus erythematosus (SLE) have increased rates of both arterial and venous thrombi [102]. In particular, APS is an autoimmune disorder characterized by recurrent thrombosis and pregnancy losses and can occur as a primary disorder or in conjunction with SLE. APS antibodies (aPL) include lupus anticoagulant, anticardiolipin antibodies, or anti-β2 glycoprotein I antibodies [103]. APS antibodies create a pro-thrombotic environment via multiple pathways, such as interference with annexin A5, an anticoagulant protein found in both adult vasculature and the placenta. The A5 protein forms a crystal that covers phospholipid membranes to prevent interaction with coagulation enzymes; however, aPLs bind annexin A5, thus inhibiting the formation of this protective shield. HCQ not only restores the original crystal layer, but also induces the formation of a second crystal layer over the anti-β2 glycoprotein I binding sites [104]. Furthermore, HCQ is capable of preventing aPL binding to the phospholipid bilayer, as well as reversing the effects of aPLs, including platelet activation, increased TF expression, increased GPIIb/IIIa expression, and increased thrombin and thrombin receptor peptide agonist generation [105][106][107][108][109][110].

aPLs may also induce thrombus formation via activation of NADPH oxidase (NOX). NOX mediates multiple inflammatory pathways, including TNFα and IL-1β signaling, and has been shown to influence endothelial dysfunction following stimulation by aPL [107][111]. The NOX ligand-receptor complex requires entrance into the endosome in order for downstream signaling to occur resulting in reactive oxygen species (ROS) production and thrombus formation. As discussed, CQ and HCQ affect endosomal function; therefore, it is no surprise that HCQ is capable of inhibiting ROS and thrombi production [112][113]. Furthermore, HCQ reverses endothelial dysfunction secondary to aPL-induced endothelial nitric oxide synthase (eNOS) inhibition and upregulation of adhesion molecules [112]. Endothelial dysfunction reversal led to decreased mesenteric thrombi in APS mice and may be mediated by HCQ activating extracellular signal-regulated kinase 5 (ERK5) [106][114]. ERK5 has been shown to have endothelial protective effects, including inhibition of leukocyte-endothelial interaction, adhesion molecule expression, and the promotion of laminar flow-induced eNOS expression [115].

Patients are often found to be hypercoagulable following a trauma. Many reasons for this have been identified including the release of platelet-derived extracellular vesicles (PEVs). Although the exact mechanism and function of PEVs is unknown, they are believed to serve initially in promoting hemorrhage control. However, persistent release of PEVs can lead to a pro-thrombotic state and increased thrombin levels. Dyer et al. demonstrated that HCQ is capable of inhibiting the release of PEVs following injury with subsequent decreased thrombus burden in a murine deep vein thrombosis (DVT) model [116].

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