Therapeutic Perspective

CLINICAL INVESTIGATION

Chloroquine enjoys a renaissance as an antineoplastic therapy

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Chloroquine (CQ) and hydroxychloroquine (HCQ), oral lysosomotropic agents with well-studied toxicity profiles, and antimalarial and antirheumatic activity, have been repurposed as antineoplastic agents based on preclinical data showing efficacy in preinvasive cancer, cancer stem cells and metastatic cancer. Phase I/II clinical trials are providing safety and efficacy data regarding CQ or HCQ monotherapy or combination therapy with molecularly targeted inhibitors in patients diagnosed with glioblastoma multiforme, breast ductal carcinoma *in situ*, non-small-cell lung cancer, hepatocellular, pancreatic, or renal cancer, multiple myeloma, or chronic lymphocytic leukemia. Disruption of autophagy-mediated cell survival is a major therapeutic rationale for using CQ and HCQ. CQ and HCQ are the first agents that rationally target cytoprotective autophagy in cancer. Short-term treatment of preinvasive breast cancer with CQ introduces the concept of preventing invasive cancer by killing preinvasive lesions.

Keywords: autophagy • breast cancer • chloroquine • DCIS • glioblastoma • hydroxychloroquine • LC3B • pancreatic cancer • radiosensitivity

Current molecular therapies for cancer, used alone or in combination with chemotherapy, are effective for only a short time period, and fail to significantly extend survival for many common cancers. Improvements in the duration of a therapeutic response have been attained by using genomic and proteomic biomarkers to guide cancer therapy. Unfortunately, these advances are too often frustrated by treatment failure due to acquired resistance driven by genetic instability or outgrowth of preexisting, therapy-resistant cancer subclones. Based on this lack of progress, there is a growing recognition that improvements in long-term outcomes for cancer will require new classes of therapy that prevent cancer by targeting preinvasive neoplastic lesions, potentiates drug efficacy or reduces drug resistance for current therapies. These new classes of therapy are envisioned to target mechanisms of carcinogenesis, tumor cell survival and cancer stem cell function that transcend or complement conventional therapeutic targets. Chloroquine (CQ) and hydroxychloroquine (HCQ), 4-aminoquinolines, are currently emerging as strong candidate drugs capable of fulfilling these broad transcendent goals of circumventing acquired drug resistance, enhancing drug efficacy and potentially preventing the transition from preinvasive to invasive and metastatic disease in select cancers (Figure 1).

In this perspective, the authors will review the growing renaissance in the clinical investigation of CQ and HCQ as antineoplastic agents for both preinvasive cancers as well as invasive and metastatic tumors. From a pharmacology perspective, CQ and HCQ are attractive agents because they have outstanding oral bioavailability and there are abundant preclinical data supporting their anticancer efficacy. CQ and HCQ:

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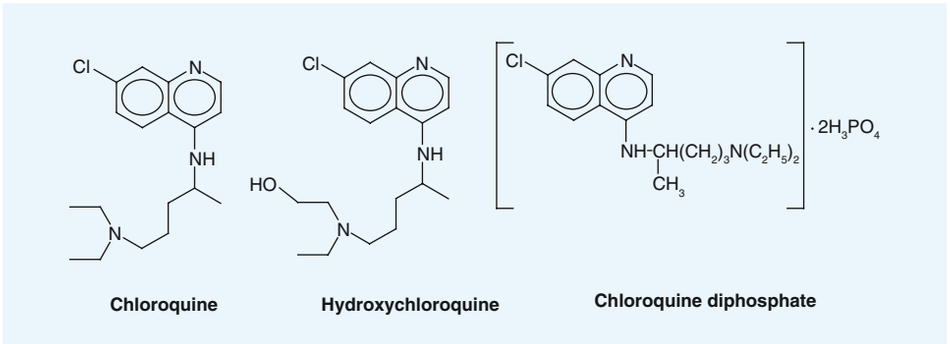

Figure 1. Chloroquine, hydroxychloroquine and chloroquine diphosphate [1,2,4,8].

- Rapidly diffuse across biomembranes to partition into acidic subcellular vesicles, such as lysosomes;
- Interfere with cytoprotective autophagy [1–4];
- Function as weak DNA intercalating agents [5,6];
- Specifically induce differentiation of tumor/progenitor cells while sparing normal cells (Figure 1) [7–9].

More importantly, CQ and HCQ are well-tolerated with a known safety and toxicity profile based on their widespread use as antimalarial and antirheumatic agents [1,2].

Repurposing CQ as an antineoplastic agent capitalizes on its broad physiological and biochemical effects related to inhibition of endosomal and lysosomal acidification [1,2,4]. Three different therapeutic approaches are currently being evaluated in Phase I/II clinical trials (Table 1) [201–235]. The first approach utilizes either CQ or HCQ as a component of combination therapies to enhance molecularly targeted drug efficacy and/or mitigate acquired drug resistance. The second approach involves enhancement of radiotherapy (radiation sensitization) following CQ or HCQ administration. CQ serves as a potentiating agent in these first two scenarios. The third approach uses a short course of CQ as a neoadjuvant/chemopreventive agent for preinvasive breast lesions [10–12]. The preclinical data and clinical trials highlighted below provide glimpses into the exciting realm of repurposing and resurrecting CQ and its derivatives as antineoplastic agents for monotherapy, combination therapy, or as a chemopreventive of preinvasive lesions (Figure 2). This review will discuss the preclinical and clinical data that support the current active interest in CQ as an antineoplastic agent. The authors will also discuss the challenges and uncertainties regarding the use of aminoquinolines in cancer. In addition, we emphasize the need for improved quantitative measures of the molecular mechanisms of efficacy of CQ and HCQ on target pathways such as autophagy.

History of 4-aminoquinilones in medicine

A historical account of malaria treatment purports that in 1630 the second wife of the Spanish Viceroy of Peru, Francesca Chinchon, was cured of malaria by a secret remedy made from the bark of the Andean Cinchona tree [13,14]. Cinchona trees comprise several species within the *Rubiaceae* family, which are indigenous to the lower altitudes of the Andes [13]. Although accurate records do not exist, the medicinal value of cinchona bark was recognized by the indigenous peoples of Peru. Cinchona bark powder was exported by the Jesuits to Europe for use as an antipyretic [13,14]. Despite malaria's eradication in most of Europe today, malaria was endemic throughout Europe, including Scandinavia in the 18th and 19th centuries. Quinine, extracted from the cinchona bark, was used to reduce malaria-related fevers [13]. Cinchona bark extract contains the alkaloids quinine, quinidine, cinchonidine and cinchonine, which produce anti-infective and antirheumatic effects [2].

High demand for cinchona bark spurred English imports of seeds and tree cuttings, which were later sold to the Dutch. Cinchona plants were successfully cultivated in Java, eventually producing the majority of the world supply of cinchona bark [13]. By the 1930s, chemists were synthesizing various forms of alkylated quinolones [13,15]. While developing more effective antimalarial agents, Hans Andersag, of Bayer AG (Berkshire, UK), synthesized CQ by modifying the acridine ring of atabrine with a quinolone ring [13,15]. CQ was initially considered too toxic for human use; however, a decade later, parallel development of the test substance SN-7619, was discovered to be the same CQ synthesized previously by Andersag [13,15]. Further structural modifications of CQ led to less toxic formulations, including HCQ and CQ diphosphate (Figure 1) [2].

Bioavailability/pharmacodynamics

Chemical structure

Structurally, CQ and HCQ differ by one hydroxyl group. CQ is usually synthesized as the diphosphate salt of N′-(7-chloroquinolin-4-yl)-*N*,*N*-diethylpentane-1,4-diamine and is a diprotic weak base (pK_{a1} = 8.1, pK_{a2} = 10.2) [4]. The molecular formula of CQ is: $C_{18}H_{26}ClN_3$, with a molecular weight of 319.92 [236]. The molecular formula of HCQ is $C_{18}H_{26}ClN_3O$, with a molecular weight of 335.87, and is also a diprotic weak base (experimentally derived pK_{a1} = 8.27, pK_{a2} = 9.67) [16].

Table 1. Clinical trials investigating the safety/efficacy of chloroquine or hydroxychloroquine monotherapy or combination therapy.

Investigative agent(s)	Conditions	Trial ID number	Phase	Location	Status	Ref.
HCQ + gefitinib	NSCLC	NCT0089237	I/II	Singapore	R	[203]
HCQ	Breast cancer, invasive (not inflammatory)	NCT01292408	II	Netherlands	R	[204]
HCQ with/without imatinib	Chronic myeloid leukemia	NCT01227135	II	UK	R	[205]
HCQ	Pancreatic cancer, metastatic	NCT01273805	II	USA	R	[206]
HCQ, paclitaxel, carboplatin bevacizumab	NSCLC	NCT00933803	II	USA	A	[207]
HCQ, capecitabine, oxaliplatin bevacizumab	Colorectal	NCT01006369	II	USA	R	[208]
HCQ + sunitinib	Solid tumors, refractory	NCT00813423	I	USA	R	[209]
HCQ	Prostate cancer, post-local treatment with elevated PSA	NCT00726596	II	USA	R	[210]
HCQ	Bone metastasis from refractory solid tumors	NCT01427403	I	USA	R	[211]
MK2206 (AKT inhibitor) + HCQ	Kidney or prostate cancer, or advanced solid tumors	NCT01480154	I	USA	R	[212]
Sirolimus + HCQ or vorinostat + HCQ	Unspecified advanced cancer	NCT01266057	I	USA	R	[213]
HCQ + radiation therapy and temozolomide	Glioblastoma multiforme, newly diagnosed	NCT00486603	I/II	USA	A	[214]
Erlotinib with/without HCQ	Chemonaive advanced NSCLC with EGFR mutation	NCT00977470	II	USA	A	[215]
Proton beam radiation, capecitabine + HCQ	Pancreactic cancer, resectable	NCT01494155	II	USA	R	[216]
HCQ + vorinostat	Advanced solid tumors	NCT01023737	I	USA	A	[217]
HCQ + sorafenib	Refractory/relapsed solid tumors	NCT01634893	I	USA	R	[218]
FOLFOX/bevacizumab + HCQ	Colorectal	NCT01206530	I/II	USA	R	[219]
HCQ + paclitaxel + carboplatin or all three + bevacizumab	NSCLC, advanced/recurrent	NCT01649947	I	USA	R	[220]
HCQ + gemcitabine/abraxane	Pancreatic cancer	NCT01506973	I/II	USA	R	[221]
HCQ + gemcitabine	Pancreatic cancer, adenocarcinoma stage IIb/III	NCT01128296	I/II	USA	R	[222]
HCQ neoadjuvant	Renal cancer	NCT01144169	II	USA	R	[223]
HCQ	Melanoma	NCT00962845	0	USA	R	[224]
HCQ, cyclophosphamide, dexamethasone, rapamycin	Multiple myeloma relapsed/refractory	NCT01689987	I	USA	R	[225]
HCQ + adesleukin	Renal cell carcinoma	NCT01550367	I/II	USA	R	[226]
HCQ + RAD001	Renal cancer, previously treated	NCT01510119	I/II	USA	R	[227]
CQ + taxane or taxotere or abraxane or ixabepilone	Advanced breast cancer, failed anthracycline	NCT01446016	II	USA	R	[228]
CQ	Breast DCIS	NCT01023477	I/II	USA	R	[229]
CQ + gemcitabine	Pancreatic cancer	NCT01777477	I	Switzerland	R	[230]
CQ + DT01 (Dbait)	Metastatic melanoma	NCT01469455	I	France	R	[231]
CQ + VELCADE + cyclophosphamide	Multiple myeloma relapsed/nonresponder	NCT01438177	II	USA	R	[232]

A: Active, not recruiting; CQ: Chloroquine ; DCIS: Ductal carcinoma *in situ*; EGFR: EGF receptor; HCQ: Hydroxychloroquine; NSCLC: Non-small cell lung cancer; R: Recruiting.

Table 1. Clinical trials investigating the safety/efficacy of chloroquine or hydroxychloroquine monotherapy or combination therapy (cont.).

Investigative agent(s)	Conditions	Trial ID number	Phase	Location	Status	Ref.
CQ + whole brain radiation therapy	Brain metastasis	NCT01727531	I/II	USA	R	[233]
HCQ + radiotherapy	Patients >70 years of age with high grade glioma	NCT01602588	II	UK	R	[234]
HCQ + sirolimus	Advanced sarcoma	NCT01842594	II	Taiwan	R	[235]

A: Active, not recruiting; CQ: Chloroquine ; DCIS: Ductal carcinoma *in situ*; EGFR: EGF receptor; HCQ: Hydroxychloroquine; NSCLC: Non-small cell lung cancer; R: Recruiting.

Bioavailability

CQ and HCQ exhibit a large distribution volume and readily accumulate in tissues such as kidney, liver, lung, spleen, muscle and melanin-containing cells in the eye and skin [17–19]. Accumulation of CQ in melanin containing cells provides a means of targeting CQ to pigmented lesions [20,21]. Cell-specific accumulation of an iodinated CQ analog (^{131}I-iodinated CQ) was shown to potentiate beta irradiation in a dog model of melanoma [21]. Tumoricidal doses of ^{131}I-iodinated CQ were concentrated within the melanin containing lesions, thus sparing the non pigmented tissues from high doses of irradiation [21].

Extensive pharmacokinetic studies reveal similarities for CQ and HCQ regarding absorption, protein binding and tissue distribution, but CQ and HCQ differ in renal clearance and elimination half-life [17–19]. Both CQ and HCQ are rapidly absorbed from the GI tract, with a mean half-life absorption of 0.57 h [17]. Bioavailability ranges from 67 to 89% [17,18]. HCQ and CQ are 50–65% protein bound, with albumin and α_1 acid-glycoprotein being the major protein-binding moieties [17,19]. HCQ stereoisomers exhibit differential protein binding, depending on the isomer and the protein [17,19]. Of note, α_1-acid glycoprotein is an acute phase reactant and CQ/HCQ binding may be affected by the increased levels of α_1-acid glycoprotein during an immune response to cancer or infectious agents [19].

CQ and HCQ can be expected to be efficacious in a variety of tissue types due to their extensive tissue distribution. Quinidine, CQ and HCQ pass Lipinski's 'Rule of 5' for qualitatively assessing the likelihood of oral drug absorption or permeation [22]. Lipinski's Rule of 5 is based on physiochemical characteristics of the compound. A drug is highly likely to be absorbed or permeate biomolecular membranes if: the relative molecular mass (M_r) is <500, the octanol–water partition coefficient (logP) is <5 (indicating lipophilicity), there are <5 hydrogen bond donors, or <10 hydrogen bond acceptors (nitrogen and oxygen atoms) [22,23].

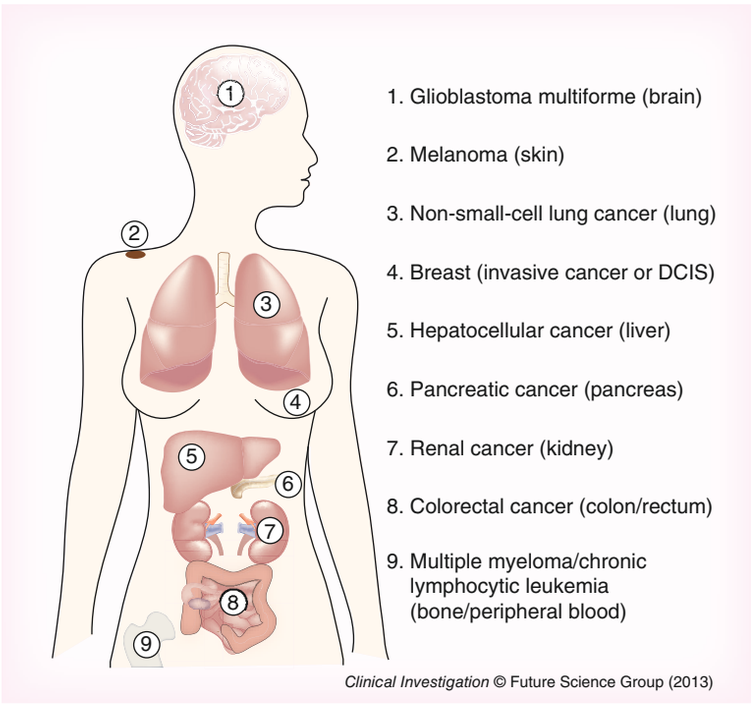

Figure 2. Target organs/conditions currently being evaluated in clinical trials using chloroquine and hydroxychloroquine as antineoplastic therapy.
DCIS: ductal carcinoma *in situ*.

Mechanism of action

CQ possesses a chlorine substitution at the seventh position of the quinoline ring [24]. Therapeutic properties of CQ are most likely mediated by the chlorine side chain structure because the quinoline ring lacks antimalarial activity [24]. CQ possesses three main mechanisms of action related to antineoplastic therapy: lysosomotropic autophagy inhibition, nonlysosomal anti-inflammatory activity and acting as a radiosensitizing agent.

Lysosomotropic autophagy inhibition

CQ is a lipophilic small molecule capable of rapidly penetrating the cellular lipid bilayer and acidic subcompartments [1,2]. As such, CQ is an effective lysosomotropic agent that reduces endosomal/lysosomal acidity [1]. This dibasic property can be exploited therapeutically to

target the acidic extracellular microenvironment of many solid tumors [17,25]. CQ and HCQ accumulate within the acidic lysosome/endosome because they become diprotonated (two positive charges) and will not diffuse freely out of these acidic compartments. CQ concentrations within lysosomes may be 10,000-fold greater compared with extracellular compartments, resulting in therapeutically obtainable ranges (millimolar) [26]. Within the lysosome, the diprotonated CQ and HCQ elevate the lysosomal pH, thus inactivating phospholipase A_1 and A_2, lysophospholipid acylhydrolase and monacylglycerol lipase [4]. Inhibition of lysosomal phospholipase A_1 promotes storage of lysosomal phospholipases [27]. Inhibition of these lysosomal enzymes hinders proteolytic degradation of lysosomal contents (Figure 3) [4]. In addition CQ and HCQ prevent fusion of endosomes and autophagosomes with lysosomes [28,29].

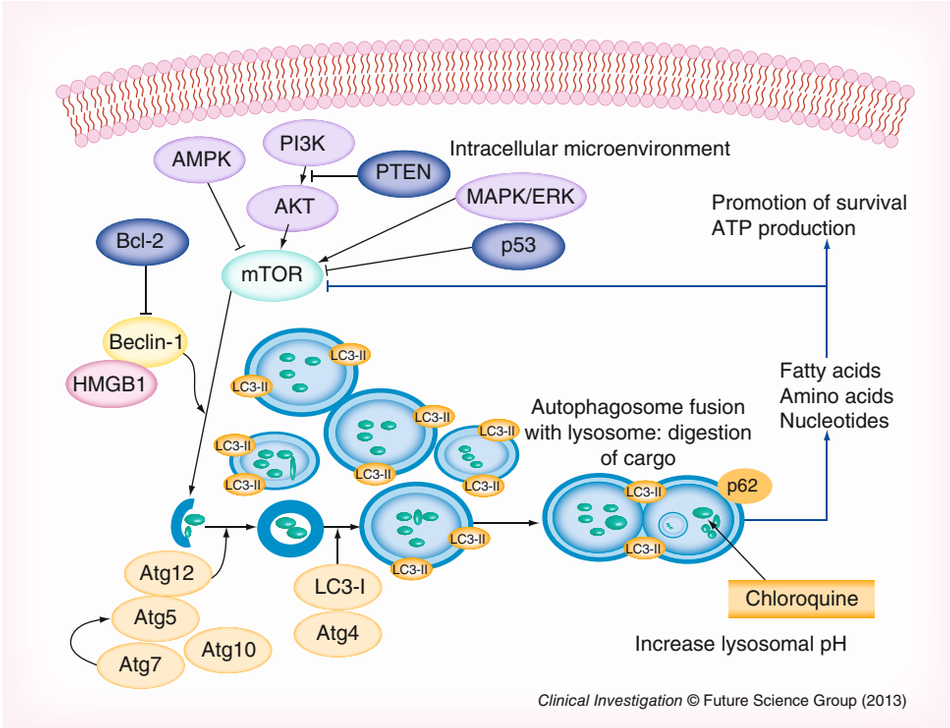

Figure 3. Autophagy is regulated via cell signaling cascades and autophagosome production. Autophagy is a feedback-loop controlled cell process of self-cannibalization that can be used to generate energy during nonlethal stress. Autophagosomes entrap cytoplasmic organelles and proteins within their double membrane vesicle, which fuses with a lysosome. Acid hydrolases within the lysosome digest the cargo, thereby releasing fatty, nucleic acids, and amino acids for recycling or energy production. Chloroquine interferes with autophagy by blocking the fusion of the autophagosome with the lysosome thus suppressing the digestion of the cargo, which elevates the cellular stress to lethal levels, ultimately resulting in apoptosis or necrosis.

In the *MYC* model of lymphoma, and other over expressed *MYC* models, the effect of CQ was not on the expression of Myc or p53 but was reported to be associated with autophagy suppression [30]. Autophagy is an evolutionarily conserved catabolic process that provides energy during periods of sublethal levels of cellular stress, or acts as an alternative cell-death pathway [3,31–33]. CQ interferes with autophagy because it raises the lysosomal pH, which inhibits fusion of autophagosomes with lysosomes, thus blocking lysosomal enzyme degradation of the autophagosome cargo (Figure 3) [28,33,34]. Over-expression of *MYC* has been thought to drive the progression of Burkitt's lymphoma and mammary carcinomas [11,30,35]. In both of these systems, suppression of autophagy has been shown to block the progression of premalignant to malignant states [30,35]. Moreover, autophagy has recently been found to be important for the survival of cancer stem cells and autophagy regulates cell 'stemness' [11,36,37].

■ Nonlysosomal mechanisms of action

CQ also exhibits nonlysosomal effects, including immune cell activation, antiretroviral activity, radiosensitization and modification of cell signal transduction [1,5,6,9,20,26,38–47]. The antiretroviral effect of HCQ in human immunodeficiency virus infection was shown to be due to suppression of HIV-1 replication by inhibiting post-translational modification of gp120 in T cells and monocytes [48].

Cell signal transduction

As a consequence of lysosomal alkalinization, several cell signaling transduction pathways dependent on phospholipid turnover have been found to be inhibited by CQ [27]. CQ alters arachidonic acid pathway signaling by inhibiting platelet phospholipase A_2 in thrombin stimulated platelets, as well as release of histamine from mast cells [27]. CQ has also been shown to block IP3 binding to its receptor, which subsequently prevents the efflux of calcium from intracellular compartments [27].

CQ is commonly used to study the role of endosomal acidification in cellular processes, such as intracellular TLR signal transduction in immune cell activation [46,49]. TLR9 recognizes, and binds regions of bacterial DNA enriched with cytosine–guanine nucleotides

(CpG) during initial innate immune responses to bacterial DNA. TLR9 mediated endocytosis of bacterial CpG-DNA, with subsequent maturation in the acidic endosome, is necessary for immune cell activation [46]. CQ was found to block TLR9 binding to CpG-DNA suggesting that CQ acts in a nonlysosomotropic manner as a TLR9 antagonist [46]. In addition, CQ mediates inflammation via reduction of TNF-α mRNA, inhibition/reduction of cytokines/chemokines and/or suppression of antigen presentation [20,26,50].

Another signal transduction effect of CQ is ubiquitin-mediated HDAC enzyme degradation with concomitant histone hyperacetylation [9,24,51]. Epigenetic histone modifications, such as acetylation/deacetylation were shown to produce a more differentiated cell phenotype in breast cancer cell lines [9,24,51].

Multidrug resistance proteins

Plasmodium falciparum resistance to CQ is associated with mutations in the parasite multidrug resistance transporter genes *pfmdr1* and *pfcrt* [52,53], which are analogs of the mammalian multidrug resistance (MDR) gene family. In *P. falciparum,* the mutated version of the transporter protein promotes CQ efflux out of the parasite digestive vacuole, effectively reducing the CQ concentration. In mammalian models, CQ has also been shown to bind to the multidrug resistance protein (MRP) in tumor cell cultures. [54]. Long-term (6 month) cell culture in the presence of CQ is associated with over expression of the MRP-1 gene [54]. Acquired resistance to CQ mediated by MRP-1 in a T-cell model has been shown to induce cross-resistance to dexamethasone, but not methotrexate, leflunomide, cyclosporine A or sulfasalazine [55]. Furthermore, mutations in the ABCR (*ABCA4*) transporter gene, a photoreceptor cell-specific ATP-binding cassette transporter gene, are associated with CQ/HCQ retinopathy [56]. Consequently, the interaction of CQ with multidrug resistance export channels may contribute to its effect on chemosensitization, and multidrug resistance-associated gene amplification in tumor cells could potentially be an adaption mechanism following long-term CQ therapy.

Radiosensitization

The mechanism of CQ-mediated radiosensitization was demonstrated to occur by three mechanisms:

- Increased lysosomal volume due to CQ accumulation;
- Mitochondrial and lysosome membrane destabilization;
- Ceramide induction through activation of acid sphingomyelinase [57].

These responses ultimately lead to necrosis from sudden release of lysosomal acid hydrolases and apoptosis by mitochondrial membrane permeability [57].

Metabolism & excretion

The metabolism and renal excretion of CQ and HCQ are stereoisomer specific. The (*R*)-stereoisomer of CQ is excreted more slowly than the (*S*)-stereoisomer [17]. The renal clearance of HCQ has been calculated as 96 ml/min, with an elimination half-life of 1200 h, whereas the renal clearance for CQ is 129 ml/min, with an elimination half-life of 288 h [17–19]. The differences in renal clearance and half-life could be critical parameters in defining dosing regimens as antineoplastic agents.

A high likelihood of absorption and permeation are essential for distribution within nonvascularized tissue compartments such as breast duct epithelium. CQ excretion in breast milk indicates that it does indeed accumulate in breast duct epithelium thus supporting its use in breast cancer and ductal carcinoma *in situ* (DCIS) [10–12,38,58]. CQ metabolism occurs in hepatocytes via the cytochrome P450 monooxygenase family [2,59–62]. CQ undergoes dealkylation to form the major metabolite, desethylCQ, which has a similar half-life as CQ [19,60,62]. Human liver microsome studies, HPLC/MS, and chemical inhibition studies identified CYP2C8, CYP3A4 as the major cytochrome oxidases responsible for CQ metabolism. CYP2D6 is also involved, but to a lesser extent [60,62]. An important consideration for combinatorial therapy is competitive inhibition of CYP2D6 by CQ [62].

Autophagy: a target for antineoplastic therapy

Autophagy regulates cell homeostasis via regulation of cell survival or cell death, via an alternative mechanism to apoptosis. First coined by de Duve, autophagy (auto-, meaning 'self', and -phagy meaning 'eating') is an evolutionarily conserved endoplasmic reticulum–lysosomal pathway response for degrading protein aggregates, and recycling nonessential intracellular organelles and cytoplasmic proteins [3,32,63–65]. Macroautophagy, herein termed autophagy, is a type of cellular self-cannibalization in which the cell sequesters organelles, such as mitochondria, or cytoplasmic proteins in double-membrane vesicles, termed autophagosomes. Basal levels of autophagy ensure removal of damaged organelles and cell survival.

However, autophagy provides a survival mechanism for cancer cells during periods of sublethal stress such as hypoxia, nutrient deprivation or detachment from their basement membrane [11,36,37,66]. Autophagy has been shown to induce surges of ATP in cultured glioma cells treated with etoposide thus promoting cell survival [67].

Autophagy disruption is thought to be a major therapeutic action of CQ. CQ and HCQ rapidly partitions into acidic subcellular vesicles, such as lysosomes [1–4], and subsequently interferes with cytoprotective autophagy (Figures 3 & 4) [33,68]. Immune modulatory effects of 4-aminoquinolines have not been completely elucidated; therefore we will focus on their lysosomotropic properties.

Autophagosomes fuse with lysosomes, wherein digestion via lysosomal acid hydrolases occurs, thus liberating energy in the form of ATP, amino acids and fatty acids. Various types of autophagy exist, defined either by the organelle involved (mitophagy, mitochondrial autophagy), by the molecular process (chaperone mediated autophagy) or by the size and location of sequestered proteins (microphagy) [68,69]. Mitophagy occurs directly on the surface of the lysosome by fusion of endocytic vesicles with the lysosome.

Autophagy is functional in a wide range of developmental and cellular differentiation process [33]. Autophagy has both cytoprotective and cytotoxic functions depending on the cell type and context of autophagy induction. In normal cells, autophagy cycles between induced or noninduced states depending on local cellular microenvironment influences. Autophagy induction occurs during cellular stresses, such as hypoxia, nutrient deprivation, oxidative stress, endoplasmic reticulum stress, or in the presence of calcium phosphate deposits within spheroids, preinvasive lesions, or solid tumors (Figure 4) [33,70,71].

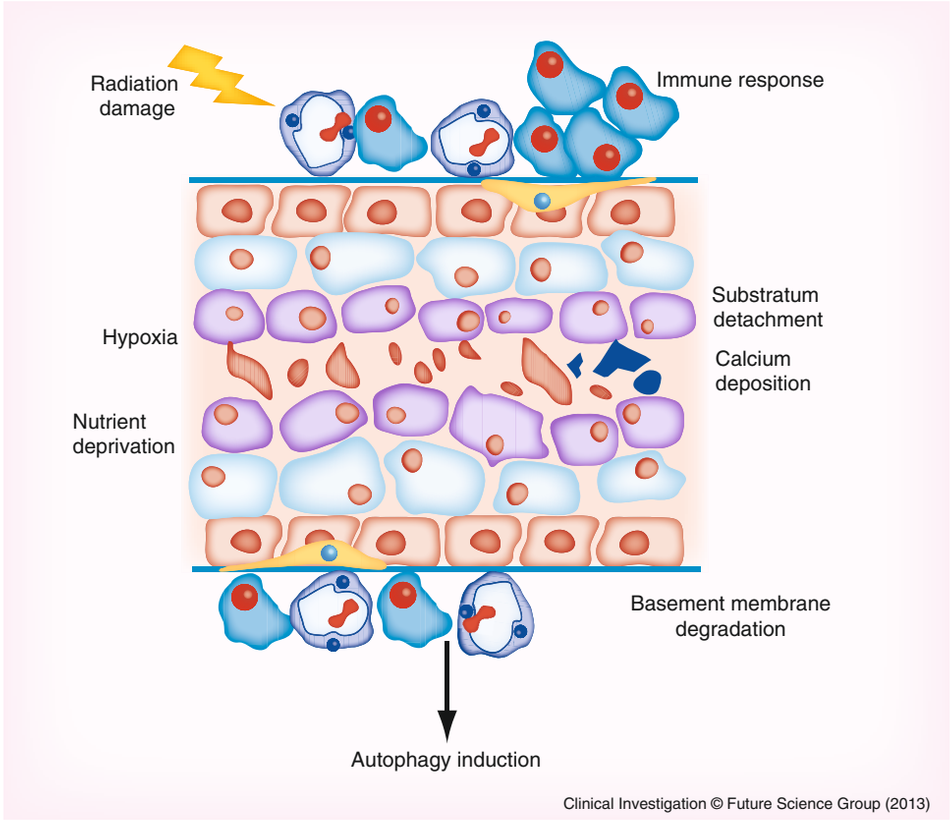

Figure 4. Inducing autophagy is a means of adapting to a high-stress microenvironment. Stem cells within spheroids, cells within areas of hyperplasia in preinvasive lesions, and cells at the core of solid tumors must adapt to a high-stress environment in order to proliferate.

Regulation of autophagy occurs at two levels: cell signaling cascades in response to extracellular or internal metabolic conditions, and autophagosome nucleation. mTOR is the central regulator of protein translation, responding to nutrient levels (amino acids) through a class I PI-3K/AKT/PDK1 protein signaling cascade. The PI-3K/AKT pathway inhibits mTOR and autophagy when nutrient levels are adequate [31]. PTEN, phosphatase and tensin homolog is a 3′ phosphoinositide phosphatase that inhibits the PI-3K/AKT pathway, thus inducing autophagy. Feedback loops through eIF2, Ras, and p70S6 kinase regulate basal autophagy (Figure 3) [31]. Endoplasmic reticulum stress, such as elevated intracellular calcium or accumulation of misfolded proteins in the lumen, can induce autophagy through reactive oxygen species and intracellular calcium-linked signaling cascades [72]. Once autophagy is initiated, further regulation occurs during autophagosome formation by cleavage of LC3-I via Atg4. Cytosolic LC3-I is lipidated by phosphatidylethanolamine on a glycine at its C-terminus, forming LC3-II, which colocalizes on the inner and outer membranes of the autophagosomes [73].

Autophagy protects stem cells from nutrient stress

Autophagy provides homeostatic control of stem cell function and is thus autophagy is an attractive target in cancer therapy. Constitutively high autophagic activity has been demonstrated in hematopoietic stem cells, dermalstem cells and epidermal stem cells [37]. In a preclinical mouse model, autophagy induction was monitored in hematopoietic stem cells derived from the bone marrow of GFP-LC3 transgenic mice [74]. Calorie restriction for 24 h resulted in decreased levels of GFP-LC3 in the hematopoietic stem cells but not the granulocyte/macrophage progenitors, demonstrating that long-lived hematopoietic stem

cells, but not short-lived progeny could induce cytoprotective autophagy [74]. Hematopoietic stem cells from *Foxo3a*$^{-/-}$*GFP-LC3* mice had twice the level of GFP-LC3 as the controls as well as a reduced capacity to induce autophagy. Hematopoietic stem cells from double knock-out *Foxo3a*$^{-/-}$/p53$^{-/-}$*GFP-LC3* mice did not show any further increases in GFP-LC3 levels or a greater delay in autophagy induction indicating that the hematopoietic stem cells used FOXO3A to maintain autophagy [74]. The fundamental mechanism of action of CQ related to cancer therapy is disruption of autophagy. Therapies that kill or abrogate the function of cancer stem cells are an emerging area in cancer treatment as well as prevention [11,36,37].

Why is CQ enjoying a renaissance as an anticancer agent?

The CQ renaissance may be attributed to several factors. CQ and HCQ are inexpensive, water soluble, orally administered, well-tolerated drugs that achieve high blood/tissue levels after a single dose [2,10,11]. These qualities are highly desirable for any drug, and are a necessity as a short-term neoadjuvant treatment or potential chemopreventive agent [10–12]. Furthermore, CQ and HCQ have well-characterized toxicity profiles based on long-term use as antimalarial and antirheumatic therapies. More importantly, CQ and HCQ are lysosomotropic agents that inhibit autophagy. Autophagy has been recently confirmed as a cytoprotective tumor strategy, a mechanism of acquired drug resistance (reviewed in [68,75]), and is functionally important in maintaining cell viability *in vivo* in breast DCIS cells [10,11].

Maintaining cellular homeostasis is an intricate balance of intermingled metabolic pathways. Within these pathways, there are many potential therapeutic interventions; however autophagy is central to the cells ability to avoid apoptosis and as a mechanism to combat hypoxia, starvation, genetic instability, and so forth, until the stress becomes insurmountable. CQ inhibits self-preservation (autophagy pathway) in stem cells, immune cells, tumor cells and preinvasive cells. Impeding self-preservation, rather than any single step of a cell proliferation pathway, should sufficiently favor destruction of the tumor cells [76]. Cells addicted to autophagy for survival undergo necrotic or apoptotic death following CQ treatment, making CQ a very promising antineoplastic candidate. Examples of the current preclinical and clinical data supporting CQ as an antineoplastic or radiosensitizing agent are discussed below.

CQ/HCQ preclinical & clinical trials

As of May 2013, 33 clinical trials using CQ or HCQ in cancer were active or recruiting (Table 1) [201–235] . CQ is being evaluated in preinvasive breast DCIS, primary and metastatic tumors.

Preinvasive lesions

Preclinical studies

Cell line models of preinvasive breast cancer have shown that aminoquinolines produce differential effects depending on the cell type/degree of differentiation [8,9,24,51,77]. Strobl *et al.* performed a comprehensive series of experiments demonstrating the differential molecular effects of quinidine on breast cell lines. Three striking observations, relevant to CQ as an antineoplastic, were:

- The differential effect of quinidine on HDAC4 levels in tumorigenic MCF7 breast cell lines compared with the immortalized nontumorigenic MCF10A breast cell line;
- Quinidine down-regulated c-Myc in MCF-7 cells but not MCF10A cells with concomitant hypophosphorylation of retinoblastoma protein;
- Quinidine caused ubiquitin-mediated degradation of HDAC, resulting in histone hyperacetylation [8,24,51,77].

In another study, Strobl and colleagues showed CQ induced DNA damage in MCF-7 cells, with subsequent stimulation of p53 and p21 [24], which was confirmed by another group in a mouse model [78]. These studies indicate that 4-aminoquinolines could have clinical utility in breast cancer based on the following evidence:

- Elevated HIF confers resistance to p53-mediated apoptosis induced by genetic damage [79];
- Abrogation of the phosphorylated retinoblastoma pathway can be a major survival strategy for breast cells by inhibiting cell cycle arrest [80];
- c-Myc, a proto-oncogene, is a transcription factor that regulates cell cycle progression and proliferation in estrogen receptor (ER)-positive and -negative breast cells, thus making c-Myc an attractive drug target for hormone-insensitive breast cancer.

Preinvasive lesions are restricted in their molecular options for survival [79–81]. Metabolic stress, nutrient deprivation, and hypoxia are known to induce mutagenesis and genetic instability, activating response programs including DNA repair, angiogenesis and autophagy [82–84]. Established tumors have other means to survive but preinvasive breast lesions are more dependent on autophagy [11]. In our preclinical studies, we established a DCIS organoid culture that spontaneously produced spheroid forming cells with stem-like properties. Treatment with CQ phosphate (50 μM) completely suppressed

the generation of DCIS spheroids, suppressed *ex vivo* invasion of autologous stroma, induced apoptosis as measured by cleaved caspases and cleaved PARP, suppressed autophagy associated proteins Atg5, AKT/PI-3K/mTOR, eliminated cytogenetically abnormal cells in the organoid culture, and prevented tumor growth in a NOD/SCID xenograft model [11].

■ Clinical trials: preventing invasive neoplasia with CQ

Preinvasive breast lesions are nonobligate precursors to invasive cancer [85,86]. Therefore a subpopulation of women with preinvasive cancer will develop invasive carcinoma. Currently, CQ diphosphate (Aralen®, Sanofi-Aventis; Paris, France) is being studied as a short-term neoadjuvant therapy in a Phase I/II clinical trial for ER-positive and -negative DCIS and atypical ductal hyperplasia (Table 1) [10–12,229].

Patients, regardless of histologic grade, are randomized to receive CQ diphosphate at one of two doses: 500 or 250 mg/week, for 4 weeks. MRI studies are performed on each patient at enrollment and prior to surgical therapy. All patients receive standard-of-care surgical therapy: mastectomy or lumpectomy depending on the size and confluence of the primary DCIS lesion. Outcome measures, post- versus pre-therapy, are:

- Reduction in DCIS lesion volume by MRI;
- Pathologic regression;
- The reduction or elimination of genetically abnormal tumorigenic DCIS stem-like cells;
- The suppression of cellular proliferation, induction of apoptosis, or disruption of autophagy, as measured by changes in proteomic markers in the post- versus the pre-treatment specimen.

Molecular measures of efficacy in the pre- versus post-treatment lesion include:

- A reduction in proliferative index by immunohistochemistry (IHC) using PCNA or KI67;
- An increase in apoptosis index by IHC using cleaved PARP and activated caspases;
- A disruption of autophagy by IHC using LC3B staining of autophagosomes [12].

The outcome of this trial should be known within the next 2 years. If the trial is successful in showing that CQ is able to reduce the radiologic size of the DCIS lesions, or reduce proliferation and/or increase apoptosis, this will set the stage for wider confirmatory studies by others. However, there is a paucity of data in the emerging field of antiautophagy therapy as a potential chemopreventive. Autophagy has been shown to have a dual role in cancer formation that may be stage and tumor-type dependent [34]. Autophagy may function as a tumor suppressor prior to invasive tumor development [35] or as a tumor promoter [34,66,87,88]. Data from ongoing clinical trials will provide much needed information regarding efficacy of antiautophagy therapies in different tumor types and stages.

Primary tumors

■ Preclinical studies

CQ and HCQ are demonstrating promise as combinatorial agents in preclinical studies of colon and melanoma cancers, as well as chronic lymphocytic leukemia [89–91].

An unintended consequence of antiangiogenesis therapy is development of hypoxia within the tumor bed. In response to hypoxia, HIF-1α is upregulated to restore oxygen hemostasis [79,84,92]. HIF-1α can subsequently inhibit p53-mediated cell death in cells with DNA damage [79]. Bevacizumab, an antiangiogenesis agent targeting the VEGF receptor, and oxaliplatin, a DNA-damaging agent, were both found to stimulate autophagy [90]. In a preclinical study assessing the utility of combinations of oxaliplatin, bevacizumab and CQ to mitigate the autophagic response, O'Dwyer and colleagues recently demonstrated that autophagy was induced by either bevacizumab or oxaliplatin in mouse xenograft tumors derived from HT29 colon cancer cell lines [90]. CQ treatment, Beclin1 shRNA interference or ATG5 down regulation, inhibited autophagy and enhanced sensitivity to oxaliplatin under normal and hypoxic conditions in a synergistic manner [90].

CQ also potentiates the cytotoxic effects of 5-fluorouracil (5-FU), an antimetabolite, anticancer drug that inhibits thymidylate synthase and ultimately DNA synthesis and repair [93]. In a mouse model of mammary carcinoma (C3HBA), a dose escalation study of 5-FU plus CQ phosphate revealed the combination therapy was extremely toxic, resulting in death of all the mice within 2 weeks, unless both 5-FU and CQ phosphate were administered in low doses (12 mg/kg 5-FU and 10 mg/kg CQ) [93]. This study highlights the potential toxicity that may arise by combining CQ with other drugs.

CCI-779, a rapamycin analog that inhibits mTOR, thus upregulating autophagy, has been approved for oral therapy of renal cancer with activation of the PI-3K/AKT/mTOR pathway [91]. CCI-779 has not shown promise in melanoma despite activation of Ras/Raf/Mek/PI-3K pathway. Xie *et al.* recognized that

autophagy was a tumor survival promoting mechanism in melanoma [91]. Combination therapy with CCI-779 and HCQ significantly reduced melanoma cell proliferation index (Ki-67) and increased the apoptotic index in mice bearing established UACC903 tumor xenografts [91].

Mahoney *et al.* recognized that endoplasmic reticulum stress induces autophagy, which supports survival of B-cell malignancies following therapy with Nelfinavir [89]. Nelfinavir, a HIV protease inhibitor that induces endoplasmic reticulum stress, is another example of repurposing a known drug for cancer therapy. Nelfinavir alone failed to cause significant cytotoxic effects in primary chronic lymphocytic leukemia cells, and increased autophagic flux. A combination of Nelfinavir and CQ significantly induced cytotoxicity, which was due to suppression of autophagy mediated survival [89].

■ Clinical trials: Phase I non-small-cell lung cancer & Phase III glioblastoma multiforme

Results from two clinical trials, in which CQ or HCQ were added to current standard of care therapies, provide evidence that CQ and HCQ are well-tolerated drugs with potentially favorable outcomes. In a Phase I study, Goldberg *et al.* explored the safety, maximum tolerated dose and pharmacokinetics of HCQ with and without erlotinib in patients with advanced non-small-cell lung cancer, stage IIIb/IV/pleural effusion [94]. In total, 27 patients were treated, eight with HCQ and 19 with HCQ + erlotinib. HCQ was safe and well tolerated and did not alter the pharmacokinetics of erlotinib. One patient in the HCQ + erlotinib arm had a partial response for an overall response rate of 5% [94]. They recommend a Phase II study dose of 100 mg of HCQ given with 150 mg erlotinib, daily for 7 days.

Sotelo *et al.* conducted a randomized, double-blind, placebo-controlled trial, adding CQ to conventional therapy for glioblastoma multiforme (GBM) [95]. The treatment cohort included 30 patients with GBM confined to one cerebral hemisphere. CQ was given beginning on postoperative day 5, in a dose of 150 mg/day for 12 months, and was compared with placebo [95]. Median survival after surgery was 11 months for controls and 24 months for CQ-treated patients. Though the authors conclude that the study is under powered to show statistical significance, the long-term survival in the CQ-treated patients justifies the expansion of a large-scale randomized trial to define the role of CQ treatment in the management of GBM [95].

Both of these small trials support the hypothesis that CQ has the potential for enhancing efficacy of conventional therapy in two types of cancer that are very resistant to treatment.

Metastatic tumors

■ Preclinical studies

CQ and HCQ have been used as therapeutic agents to treat metastatic cancer. The action of these compounds on established metastatic colonies in experimental models may be due to the effect on autophagy and cancer immune surveillance. High-dose IL-2 exhibits antitumor effects in renal carcinoma and melanoma by enhancing cytolytic immune cell proliferation [20,42]. However, IL-2 is associated with severe toxicity via a cytokine storm and systemic autophagy syndrome [20,42], thereby greatly limiting its therapy potential. IL-2 activates autophagy through classic autophagy/stress-related cell signaling cascades – Ras, PI3K, AKT, the Janus associated kinases (JAK1–3), and STAT5 (a transcription factor). IL-2 activates natural killer cells and promotes maturation of regulatory T cells. In addition, IL-2 released HMGB1 into the serum, which may be related to the initiation of systemic autophagy syndrome [20]. This global stress response promotes 'immune cell-mediated autophagy', which is cell contact dependent and contributes to vascular leak and organ dysfunction [20,42].

Using a murine model of metastatic colorectal cancer or pancreatic adenocarcinoma, Lotze and colleagues demonstrated that CQ dampens the cytokine storm after high-dose IL-2 but also enhances tumor apoptosis, and overall murine survival (>150 days) [20,42]. IL-2 and CQ co-administration augmented natural killer cell infiltration into the murine metastatic tumor [20,42]. Of note, CQ administration in this murine model decreased levels of all cytokines except IL-8 [20]. This preclinical metastasis model provides data supporting HCQ as an immune modulator via suppression of IL-2 in pancreatic cancer, melanoma and renal cancer clinical trials.

Radiation sensitivity

At least three molecular pathways affect radiation sensitivity: DNA repair, chromatin remodeling and plasma membrane cell signaling [96]. Radiation sensitivity is a complex interplay between the rate and fidelity of double-strand breaks, spatial distribution of chromatin, acetylation of histones, and upregulation of alarmin proteins or reactive oxygen species [20,42,96]. Acetylated histones 'open' chromatin, facilitating access to chromatin remodeling complexes, transcription factors and DNA repair enzymes, thus attempting to counteract the effects of the damage [97]. Ceramide release from the plasma membrane acts as a transducer of SAPK/JNK inducing a proapoptosis cascade [96]. Other cellular proteins/underlying genetic mutations contribute to a cell's ability to respond to DNA damage. Mutated *TP53* contributes to delays in inducing apoptosis. EGF receptor activation by reactive oxygen species inactivates protein tyrosine phosphatases, shifting the equilibrium to more

phosphorylated EGF receptor residues, which in turn initiates a prosurvival downstream signaling cascade through mitogen activated protein kinases [96]. This complex system of checks and balances, along with the influence of underlying genetic mutations, may partially explain the lack of consensus in the literature regarding CQ's ability to enhance/diminish radiation sensitivity [39,57,98–101].

Preclinical studies of radiosensitivity

Autophagy induced by radiation may be protective and suppression of autophagy by CQ may mediate radiosensitivity [39,101]. In ER-positive, wild-type p53, ZR-75-1 breast cancer cell lines, calcitrol plus irradiation failed to induce significant apoptosis. Autophagic flux at 72 h postirradiation was confirmed by p62 degradation, suggesting a cytoprotective effect from autophagy [101]. CQ treatment decreased the number of viable cells, mitigating the protective effect [101]. Clinically these results imply that CQ treatment would be expected to be beneficial for DCIS patients treated with CQ who receive follow-up radiotherapy [10,12]. However, because CQ accumulates in melanin-containing cells, radiosensitivity could nonetheless occur in darkly pigmented individuals. In the anecdotal report by Rustogi *et al.*, a patient being treated for a brain tumor with external beam radiation was started on a regimen of CQ as malaria prophylaxis. The patient developed a desquamating lesion, localized to the site of radiation therapy [45,102]. Discontinuation of CQ promoted healing, suggesting that CQ contributed to the sudden on-set radiosensitivity.

Class I PI3Ks regulate cell growth and survival through an AKT/mTOR cascade, thus making this pathway an attractive target for molecularly targeted therapies. NVP-BEZ235 (Novartis, Basel Switzerland) is a dual PI-3K/mTOR inhibitor currently being evaluated in Phase I radiosensitization trials. NVP-BEZ235 abrogates phosphorylation of ATM and PKC proteins in response to radiation, providing a degree of radiosensitivity. Split-dose radiation schemes allow cells time to repair DNA damage. Pretreatment with NVP-BEZ235 decreased the number of viable cells following split dose radiation [99]. However, NVP-BEZ235 also promotes autophagy [99]. CQ treatment of SK20B cells, from a head and neck squamous cell cancer cell line, further enhanced the cytotoxic effect of NVP-BEZ235 + irradiation [99]. Another study demonstrated cytotoxic effects on radioresistant stem-like glioma cells and spheroids using a triple combination of γ-irradiation, CQ and PI3K inhibitor PI-03 (pyridinylfuranopyrimidine compound) [103]. This triple combination was effective at lower irradiation and inhibitor doses (3.5 Gy, 5.0μM CQ and 5.0μM PI-103) compared with monotherapy or double-therapy suggesting that combination therapy may provide less overall toxicity to the patient [103].

Clinical trial evaluation of radiosensitivity

A fascinating Phase I trial in France for metastatic melanoma combines CQ with a small molecule mimicking DNA double strand breaks [231]. The novel small molecule, Dbait (DNA Therapeutics; Paris, France), is an oligonucleotide that traps the DNA-dependent protein kinase (DNA-PK) complex rendering it hyperactive [104–106]. DNA-PK and ATM are recruited to sites of DNA damage and hyperactivation of DNA-PK by Dbait causes hyperphosphorylation of histone H2AX. H2AX phosphorylation indicates sites of DNA damage, and once it is phosphorylated it prevents further detection of DNA damage [104–106]. The rationale for a trial combining Dbait with CQ is enhanced radiosensitization by the Dbait + CQ combination.

The caveat to these, and other preclinical studies, is that *in vitro* models fail to recapitulate the intricate *in vivo* microenvironment; however ongoing clinical trials will reveal the true *in vivo* outcomes [39].

Challenges/unmet needs

There are many challenges to overcome before CQ and HCQ are successfully translated into routine use in clinical oncology. The first set of challenges surround the choice of compound type and treatment regimen, and the adoption of quantitative measures of therapeutic outcome. Assuming that efficacy is successfully demonstrated by current clinical studies for some cancer indications, validation studies by independent clinical groups will require adequate funding. Consequently, the second set of challenges involves the resources required to support the development of a repurposed drug that does not have a pharmaceutical company champion.

Quantitative measures of outcome

The challenges for interpreting clinical trial results related to CQ-based therapeutic efficacy include:

- Extrapolating data between different 4-aminoquinoline compounds;
- Quantitatively measuring efficacy by imaging or molecular end points of proliferation, apoptosis or autophagy;
- Judging the impact of the physiological tissue context and the cancer stage on the treatment outcome.

The first challenge is that different types of aminoquinolines are being studied in clinical trials and the conclusions from one type may not necessarily translate to the other types. Therefore the specific 4-aminoquinoline administered in the trial must be specified because each compound has unique pharmacodynamic parameters [1,2,8]. Pharmacokinetic/dynamic properties of 4-aminoquinoline compounds have been extensively

studied *in vitro,* demonstrating the differential effects of the compounds based on cell type [4,8,24]. In a series of cell differentiation assays with breast cancer MCF-7 cell lines, the quinolone ring failed to induce differentiation [8]. The quinoline ring itself lacks antimalarial activity, thereby inferring that the quinoline ring is not the active structure for autophagy inhibition [24]. Strobl's group reported that the functional capacity to suppress the cell cycle proliferation protein E2F1 was a common feature of the 4-aminoquinoline compounds (CQ, quinidine, and quinine) [24]. These three compounds demonstrated antiproliferative activity, but they exhibited variable capacities to induce apoptosis and DNA damage in tumorigenic MCF-7 and non-tumorigenic MCF10A cell lines [8,24]. In a preclinical breast cancer model, CQ and HCQ inhibited ER-positive MCF-7 cells and ER-negative MDA-MD231 cells at significantly lower IC_{50}/IC_{25} concentrations compared to MCF-10A cell lines [9]. In addition, the $IC_{50}/IC_{25}$of CQ was lower (33 ± 1.5/14) for MCF-7 cells compared to HCQ (57 ± 1.3/30). These studies clearly show that the subtle structural differences between CQ and HCQ produce varying degrees of biological response based on the compounds physiochemical properties.

The second challenge will be dissecting the physiological effect of CQ and HCQ treatment at the pathologic and molecular level. CQ or HCQ treatment efficacy against neoplastic cells can be due to effects on both the host and the tumor. Efficacy can be mediated by a variety of mechanisms that directly or indirectly effect tumor cell survival and proliferation, immune cell function, and sensitivity of the host and the tumor to radiation or chemotherapy. Standardized protocols exist for measuring tumor proliferative index by Ki-67 or PCNA IHC, and the measurement of tumor volume change by imaging. For CQ and HCQ treatment, the major challenge is how to monitor autophagy. Disruption of autophagy-mediated cell survival is a major therapeutic rationale for using CQ and HCQ. Therefore, investigators are striving to employ measures of autophagy as surrogate end points of therapeutic outcome in patients. In normal cells, autophagy is a transient, highly regulated, complicated cascade that is used for survival in nonlethal stress conditions. As exemplified by hematopoietic stem cells and granulocyte/macrophage progenitor cells, autophagy upregulation is cell type and context dependent [74], and basal autophagy has a variety of physiologic roles in health and disease. The duration of this dynamic, bivalent process also depends on cell type, cell context, acidity of the microenvironment and epigenetic state of the cell [20,32,33,66,68,75,107–111]. The questions are: 'how do we measure autophagic flux?' and 'in which cell types do we monitor autophagy?'

If we wish to measure autophagy in a tissue sample, what are normal autophagy values? Basal autophagy is not completely understood. Nor are there standardized assays for measuring autophagic flux in fixed tissues or *in vivo* [68,69,89,111,112]. Some investigators have measured LC3B staining by IHC or immunofluorescence to mark the autophagosomes and thereby reflect the state of autophagic flux. Unfortunately, total levels of LC3 may not necessarily change in a predictable manner. Several subspecies of LC3 change depending on the rate of autophagy induction upstream and the function of lysosomal degradation downstream. There may be increases in the conversion of LC3-I to LC3-II, or a decrease in LC3-II relative to LC3-I, if degradation of LC3-II via lysosomal turnover is particularly rapid. The ratios of basal and reactive LC3 species can be cell type specific. In cells of neuronal origin a high ratio of LC3-I to LC3-II is a common finding [69]. SH-SY5Y neuroblastoma cell lines display only a slight increase of LC3-II after nutrient deprivation, because they may have a high basal level, whereas LC3-I is strongly downregulated, although cell specific differences in transcriptional regulation of LC3 may also play a role [69]. The pattern of LC3-I to LC3-II conversion seems not only to be cell specific, but also related to the kind of stress to which cells are subjected. To make matters more complicated, treatment with CQ or HCQ may disrupt autophagy function but may at first increase the accumulation of autophagosome-marked LC3B species caused by CQ or HCQ disrupting the lysosomal fusion with the lysosome, and the subsequent degradation of LC3. The CQ- or HCQ-treated cell can become constipated with autophagosomes and this state can persist until the cell either down regulates autophagy or goes into apoptosis or senescence.

Autophagy may promote the survival of tumor cells in one context but in another context, autophagy may contribute to tumor cell death [20,32,33,66,68,75,107–111]. Basal autophagy promotes tumor suppression most likely via removal of damaged organelles or by regulating degradation of p62, an autophagy adaptor protein [68]. How long does autophagy last *in vivo*? What effect do autophagy activating mutations, such as oncogenic *RAS* mutations, have on CQ treatment *in vivo*? Should CQ or HCQ be administered intermittently at high doses or at low doses for a longer duration? The trade-off in using CQ to eradicate cancer cells is that the cell context and timing are critically important in shutting-down aberrant autophagy, but not diminishing basal functions [68,113]. A potential solution to address the problem of unknown basal autophagy levels in human fixed tissues is to examine the same patient's tissue before and after treatment as a means for assessing the *in vivo* molecular effects of CQ.

■ Effect of stage, tissue context & duration of treatment

Autophagy disruption by CQ or HCQ may have a completely different effect on the cancer depending on the disease stage [34,66]. Neoplastic cells may be dependent on autophagy for survival within physiologic tissue niches, such as the hypoxic, nutrient-deprived breast duct where atypical ductal hyperplasia or DCIS are spawned. Administration of CQ and HCQ monotherapy at the preinvasive stage of cancer progression, when the neoplastic cells or the emerging cancer stem cells are addicted to autophagy for survival, or at the time of the early metastasis when the cells are detached from the substratum and migrating within the stroma, may be successful [10,11]. On the other hand, a highly neovascularized primary or metastatic tumor may not require autophagy for survival. For example, Takamura *et al.* reported that autophagy-deficient mice develop multiple liver tumors, while Wei *et al.* concluded that suppression of autophagy inhibits mammary gland tumorigenesis in mice [35,88]. Consequently, CQ and HCQ monotherapy may have marginal effects on established primary tumors and metastatic colonies. For vascularized tumors, autophagy may play an important role in the acquisition or emergence of resistance to therapy. Therefore, CQ and HCQ may be much more effective as potentiating or enhancing agents in combination with other therapies for these stages of cancer. Nevertheless, optimizing combination therapy dose and treatment schedules can only be based on scant empirical data. The optimal duration of autophagy inhibition is currently unknown, particularly when used in combination with DNA damaging agents and molecular targeted inhibitors. The ability of 4-aminoquinolines to intercalate with DNA could potentially increase apoptosis in normal cells, although this seems unlikely based on the long-term use of CQ as antimalarial and antirheumatic agents [5,6]. Dissecting the specific beneficial or toxic contributions of each drug in multiregimen clinical trials could be challenging owing to potentially unknown drug interactions, tumor heterogeneity, tumor genetic instability and comorbidity in each patient [76,114]. Nevertheless, as these issues are being sorted out, the advantage of CQ and HCQ is that a wealth of pharmacokinetic/pharmacodynamic data exist for 4-aminoquinilone compounds administered to patients with malaria, systemic lupus erythematosus, rheumatoid arthritis or HIV, which may help elucidate specific mechanisms of action in cancer patients [2,17,38,60–62,115].

■ Impact of potential immune suppression

CQ has been shown to be potentially beneficial for the treatment of autoimmune and inflammatory disease including lupus, arthritis and sarcoidosis [26,50]. Immunomodulation by CQ has been suggested to be due in part to reduced production/response to cytokines/chemokines such as interleukins (IL-2 and IL-6) and TNF-α [42,50]. The chemokine CXCL12 binds to its receptor, CXCR4, initiating downstream effector pathways. CQ and HCQ were identified via *in silico* modeling to be a potential antagonist to CXCR4-mediated cell proliferation [41]. Confirmatory studies in pancreatic cancer cell lines revealed that CQ and HCQ effectively antagonized CXCR4-mediated cell proliferation. Furthermore, pretreatment of pancreatic cell lines with CQ and HCQ increased apoptosis, thus supporting the use of CQ to modulate inflammatory cell responses [41].

During the long-term treatment of sarcoidosis by high dose CQ it was concluded that the effects of CQ were mainly anti-inflammatory and the action of CQ was very dissimilar to corticosteroids [50]. CQ has been shown to inhibit lipopolysaccharide induced *TNF*-α gene expression by a non-lysosomotropic mechanism [26]. CQ also antagonizes the immunostimulatory CpG-oligodeoxynucleotides, thereby promoting apoptosis of B cells [43].

The reduction of inflammation is thought to be beneficial to the cancer bearing host. Nevertheless, it is unclear how much of the anticancer effects reported for CQ in published clinical trials are influenced by the effect of CQ on the immune system. In some current ongoing clinical trials CQ is administered in conjunction with immunosuppressive chemotherapy. For these trials, and others planned in the future, it will be important to monitor parameters of immune function, because there is the potential that CQ can accentuate other immunosuppressive therapies.

■ Funding for CQ/HCQ validation trials

An often unstated challenge for clinical trial development is funding, particularly for validation trials that are not supported by the NIH or other funding sources. From a pharmacoeconomics standpoint, it appears that CQ and HCQ have a high benefit–cost ratio because these drugs are off-patent, widely available and inexpensive. While these features make CQ and HCQ highly desirable from the patient and clinician's perspective, the low profit margin may deter pharmaceutical companies from sponsoring clinical trials with CQ monotherapy. Hopefully the results of the ongoing clinical trials will provide sufficient efficacy data to encourage pharmaceutical collaborations with entities using CQ and HCQ in combination with specific proprietary drugs [116].

Future perspective

The fact that a number of antineoplastic treatment trials using CQ/HCQ are presently underway has

many significant implications for the future of cancer therapy. First, these trials will stimulate the growing interest in repurposing drugs for antineoplastic use. The advantage of repurposed drugs is that we already know their pharmacodynamics, their toxicity and often their mechanism of action. CQ is just one of many repurposed agents being considered for antineoplastic use; additional agents include Metformin and Celecoxib [237]. A further advantage will be a positive impact on healthcare costs for patients and the medical establishment. Repurposed drugs will have known, or lower toxicity, with markedly reduced cost per dose.

Although 4-aminoquinolines hold promise as antineoplastic agents, modifications of their molecular structure, or different compounds altogether, may be better suited for combination therapy or monotherapy [117–119]. Since CQ was first discovered a large number of sophisticated approaches exist for designing orally available agents with optimal physiochemical properties [23]. We can imagine new classes of antineoplastic drugs that are derived from CQ, which are independent from its role as an antimalarial agent.

A second impact of CQ/HCQ anticancer trials will be to recognize and promote the importance of targeting the inhibition of autophagy for premalignant cancer therapy, or for reducing cancer drug resistance. In the past, many oncologists have confused autophagy with apoptosis. In contrast to apoptosis, autophagy is now recognized as a sophisticated survival and quality assurance mechanism, not just another death pathway. Positive clinical data with CQ/HCQ can lead to more efforts to understand the role of autophagy in cancer and may encourage the discovery of new drugs that modulate specific components of the autophagy cascade.

A further impact of CQ/HQ antineoplastic therapy will be on the field of cancer stem cells. A series of published studies have now confirmed that spheroid forming cancer stem cells may require autophagy for survival, tumorigenicity and invasion [11,91,103]. Targeting autophagy to treat cancer stem cells alone or in combination with other emerging therapies can provide a truly fresh approach to cancer therapy. This approach has the potential to suppress cancer stem-like cells as they originate in preinvasive lesions [11]. Moreover, the survival of cancer stem-like cells following traditional therapy is hypothesized to be the basis of post-treatment recurrence and metastasis dormancy. Consequently, short-term antiautophagy therapy in combination with other therapies may increase the durability of new treatments.

A final impact of these trials can be in the field of cancer prevention. In the next 10 years, the potential exists for new types of cancer prevention therapies that kill or suppress preinvasive lesions. CQ may be one of the first of these new classes of oral therapies that selectively kill or suppress genetically abnormal cells that are precursor lesions, or emerging cancer stem cells, that drive invasive cancer. The molecular basis supporting CQ cancer chemoprevention is based on its anti-inflammatory and antiautophagy actions. Spheroid forming cancer stem-like cells, preinvasive cells, and early stages of carcinomas and lymphomas have been shown to utilize autophagy to survive and proliferate in their respective high-stress microenvironments [11,36,37].

Moving further into the future, we can imagine combination prevention therapies with CQ and other low toxicity agents that suppress multiple cellular survival mechanisms used by preinvasive lesions, for any type of cancer. Therapy aimed at killing the preinvasive lesions does not have to target the neoplastic cells only. The cellular targets can be:

- The immune cells that often infiltrate the stroma adjacent to the preinvasive lesion;
- The stromal fibroblasts and vascular cells;
- The stromal mesenchymal stem cells.

All of these cell types may nurture the expansion and survival of preinvasive lesions. The concept of cancer prevention extends to the prevention of lymphatic or blood borne metastatic disease, where CQ can prevent the survival or augment the immune surveillance of colony forming cancer cells at distant sites. Despite this promise, there is much work to be done, because the potential chemoprevention or chemotherapeutic action of CQ (alone or in combination therapy) may be highly dependent on the type and stage of cancer, the tissue context, and the duration and dose of treatment.

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Executive summary

History of 4-aminoquinolines in medicine

- Chloroquine (CQ) and hydroxychloroquine (HCQ; 4-aminoquinolines) are oral, well-tolerated lysosomotropic agents with well-characterized toxicity profiles as single agents, and are clinically well established as antimalarial and antirheumatic drugs.
- Phase I/II clinical trials are providing safety and efficacy data regarding monotherapy or combination therapies of CQ or HCQ and molecular targeted inhibitors in patients diagnosed with glioblastomamultiforme, non-small-cell lung cancer, hepatocellular carcinoma, pancreatic cancer, renal cancer, chronic lymphocytic leukemia or multiple myeloma.
- CQ and HCQ have recently been repurposed as antineoplastic agents for monotherapy or combination therapy based on preclinical data showing efficacy in preinvasive breast cancer, cancer stem cells and metastatic cancer.

Bioavailability/pharmacodynamics

- CQ and HCQ have low toxicity and excellent oral bioavailability. CQ and HCQ, weak diprotic bases, rapidly diffuse across plasma membranes and accumulate in tissues, providing easily obtainable therapeutic levels.

Autophagy: a target for antineoplastic therapy

- Disruption of autophagy-mediated cell survival is a major therapeutic rationale for using CQ and HCQ. CQ and HCQ disrupt autophagy and are the first agents that rationally target this process in cancer.
- Radiation sensitivity due to CQ and HCQ is being evaluated *in vivo* and may be dependent on cellular context. Phase I/II clinical trials are ongoing.
- 4-aminoquinolnes may exert a cytotoxic or cytoprotective effect depending on the cell context, underlying oncogenic transformations, and metabolic/environmental stress level. The promise of these agents must be tempered with the recognition that little is known about their dosage in combination with molecular targeted agents or radiation, and how best to monitor their efficacy at the molecular level.
- Short-term CQ treatment of preinvasive breast cancer introduces a new paradigm for breast cancer chemoprevention: preventing cancer by killing preinvasive lesions.

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