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Dissecting pharmacological effects of chloroquine in cancer treatment: interference with inflammatory signaling pathways

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Summary

Chloroquines are 4-aminoquinoline-based drugs mainly used to treat malaria. At pharmacological concentrations, they have significant effects on tissue homeostasis, targeting diverse signaling pathways in mammalian cells. A key target pathway is autophagy, which regulates macromolecule turnover in the cell. In addition to affecting cellular metabolism and bioenergetic flow equilibrium, autophagy plays a pivotal role at the interface between inflammation and cancer progression. Chloroquines consequently have critical effects in tissue metabolic activity and importantly, in key functions of the immune system. In this article, we will review the work addressing the role of chloroquines in the homeostasis of mammalian tissue, and the potential strengths and weaknesses concerning their use in cancer therapy.

Keywords: Autophagy; chloroquine; drug repurposing; inflammation; lysosome; neoplasm.

Introduction

The chloroquine family of drugs (CQs), such as chloroquine (CQ) and hydroxychloroquine (HCQ), are 4-aminoquinoline-based compounds that have been used for the prevention and treatment of malaria. They have also been used as anti-inflammatory agents for treating rheumatoid arthritis, and lupus erythematosus.[1] CQ is one of the most prominent cases of drug repurposing in cancer[2] and it was introduced as an auxiliary anticancer agent more than a decade ago.[3] In preliminary research, CQ and HCQ have shown promising results in combination with other anticancer drugs.[4,5] Even though CQs have shown the potential to affect cell invasion, chemotaxis, trans-differentiation, and clonogenicity in cancer research, they are mainly used as inhibitors of autophagy, which is a collective term for diverse mechanisms for intracellular degradation of macromolecules and organelles through lysosomes[6] (Fig. 1a).

Autophagy is an intrinsic cellular mechanism by which cells degrade and recycle their dysfunctional components through lysosomes. Contrasting roles of autophagy have

Abbreviations: AMPK, adenosine monophosphate-activated protein kinase; ATG4C, autophagy related 4C cysteine peptidase; ATM, ataxia telangiectasia mutated kinase; BET, bromodomain and extra terminal domain; CIC, cancer-initiating cells; CDK, cyclin-dependent kinase; CQ, chloroquine; CSC, cancer stem cells; CXCL, C-X-C motif chemokine; CXCR, C-X-C chemokine receptor type; DNA-PK, DNA-protein kinase; GR, glucocorticoid receptor; HCQ, hydroxychloroquine; IDH, isocitrate dehydrogenase; Ig, immunoglobulin; IFN, interferon; IκB, inhibitor of NF-κB; IL, interleukin; MHC-I, major histocompatibility complex class I; NK, natural killer; NF-κB, nuclear factor κB; NRF, nuclear factor erythroid-related factor; Par-, prostate apoptosis response-; SIRT, sirtuin; SQSTM, sequestosome; STAT, signal transducer and activator of transcription; TFEB, transcription factor EB; Th, T-helper; TLR, Toll-like receptor; Treg, regulatory T cell; TRAF, tumor necrosis factor receptor-associated factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; UPS, ubiquitin-proteasome system

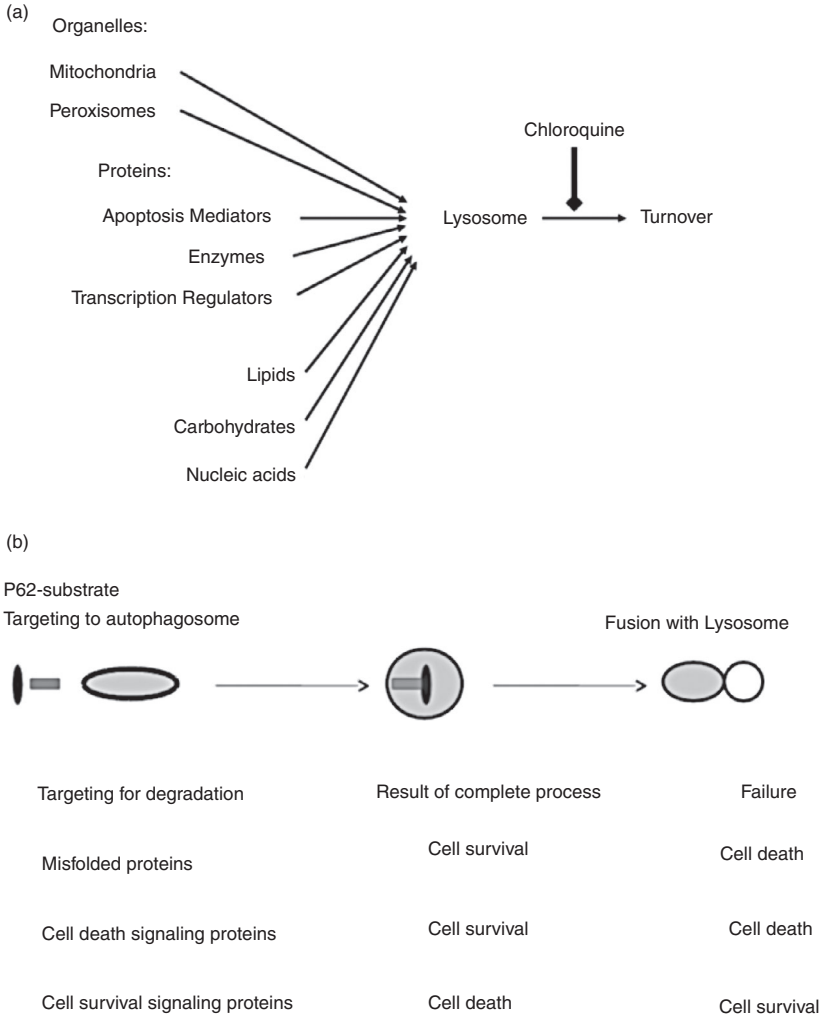

Figure 1. (a) Depending on the cellular growth conditions, diverse macromolecules and organelles such as mitochondria and peroxisomes are selectively delivered to lysosomes to be degraded through autophagy. Autophagy enables cellular adaptation to increased mutagenic load or to changes in nutrient concentrations. Chloroquine blocks lysosome acidification and thereby inhibits degradation of macromolecules and organelles by lysosomes. (b) The process of autophagy commonly involves a complex series of molecular modifications that leads to the formation of the autophagosome. Docking of adaptor proteins such as p62 on the autophagosome is followed by fusion of the autophagosome with a lysosome. Depending on the identities of the molecules degraded by autophagy, the process may lead either to cell survival or cell death. These processes are targets of chloroquines (CQs), which thereby lead to cell death or cell survival by interfering with the functions of lysosomes

been reported in cancer cells.[7] It may function as a tumor suppressor mechanism in the cells by degrading damaged proteins and organelles and therefore preventing their accumulation in the cells.[8] However, it can also promote cell survival by recycling intracellular organelles and proteins in tumors.[9] Hence, autophagy is considered a mechanism potentially leading to invasion, chemotaxis, transdifferentiation, and clonogenicity of tumor cells.

Autophagy may be selective or non-selective. In selective autophagy, substrates are selected for degradation by binding to specific adapter proteins, and the identity of the degraded molecules determines the impact of autophagy on cell fate.[10] The outcome of autophagy, therefore, becomes an important variable and a criterion in the evaluation of the effects of CQ on cancer cells. Several clinical trials that have included CQ or its analogs in combination with other anticancer agents have been initiated and the results of a number of them have been evaluated, with inhibition of autophagy as one of the outcome measures[11,12] (updated information is available at https://clinicaltrials.gov/ct2/results?cond=cancer&term=chloroquine&cntry=&state=&city=&dist=). It is important to note that the inhibition of autophagy is a crucial factor for the success of radiation therapy as well. It is known that radiotherapy leads to autophagy in the cells, which results in radioresistance, at least in glioblastoma.[13] Therefore, CQ can also sensitize tumor cells to radiotherapy by inhibiting autophagy.[14] This event was shown in

various cancer cells including glioma, breast and lung cancers.[15] In addition, CQ increased the survival time of glioblastoma patients.[16] Overall, meta-analysis shows that addition of CQs to chemotherapy or radiotherapy regimens increases overall survival and progression-free survival.[17]

Overview of the strengths and weaknesses of CQ as a cancer drug

Chloroquine is on the World Health Organization's Essential Medicine list, and was a widely used medicine for decades without any major side effects. This, and its promising preclinical results, make CQ an outstanding candidate for repurposing in cancer treatment.[1,6,18] The Repurposing Drugs in Oncology project that focuses on repurposing some well-known and well-characterized non-cancer drugs for new uses in cancer treatment, has emphasized the importance of CQ and HCQ in sensitizing cancer cells to various cancer treatment protocols.[12,19] The easy synthesis of quinolines with selected chemical properties and their environmental sustainability[20–22] are added bonuses for their use in research and clinics, if proven to be effective anticancer drugs.

A summary of past and ongoing clinical trials for CQs in cancer is given in Table 1. As expected of drug repurposing studies, the majority of trials are sponsored by hospitals/educational institutions. It is encouraging to note the increasing number of studies that combine CQ or HCQ with both established as well as novel methods of intervention, to explore concepts of synthetic lethality. Furthermore, it is understandable that a number of factors that include the legitimate concern for potential side effects may slow the pace of patient accrual. On the other hand, it would be desirable to see an increase in collaborative studies, and an improvement of the immunological approach of CQ use. In this direction, progress in drug delivery methodologies can help to focus the impact of CQ-induced cellular stress on the components of the immune system that generate the fewest side effects. Increasingly sophisticated incorporation methods for CQ in protocols for cancer immunotherapy may benefit patients with co-morbidities that cannot be included in trials without a better understanding of the immunological effects of CQs. The development of CQ-incorporating immunotherapy schemes may require the design of parallel *ex vivo* research models to accompany clinical trials. Overall, clinical research for CQ delivery methods and their incorporation into cancer immunotherapy, by lowering the required CQ concentration, will help to address the side effects that occur due to the use of high CQ doses for prolonged time periods.

It is noteworthy that in addition to their antitumor effects, CQs also have some general clinical benefits. For example, HCQ shows a tendency to improve the metabolic, especially lipid, profile of diverse groups of patients.[23,24] Indeed, it was shown that the use of CQs improved lipid profiles of individuals with rheumatoid arthritis.[25] Furthermore, CQ can protect mammalian tissues such as liver and bone marrow from the damaging effects of other drugs and irradiation.[26] Consistently, CQ and HCQ can also protect skin cells against ultraviolet light damage.[27] In further support of arguments for the study of innovation in drug repurposing, cancer is a global disease and the world's aging population will be in need of economically and environmentally sustainable cancer treatment options.[28,29]

However, despite a wealth of preclinical experimental data on the beneficial impact of CQ on malignant tumors, clinical studies have not yet shown a substantial response of neoplastic diseases to CQs.[6] There are two main potential reasons for this delay in the translation of experimental results into clinical results. The first is the inherent difficulty of CQ to pass through the cell membrane in the presence of an acidic extracellular microenvironment.[30] The tumor microenvironment is often acidic, in contrast to normal tissue that, at least under homeostatic conditions, is mostly slightly alkaline.[31] The acidity of the tumor microenvironment is mostly due to the 'Warburg effect' in cancer cells, which is the use of glycolysis rather than aerobic respiration for energy production.[32] Compared with the normal cells, cancer cells use more glucose to sustain a high proliferation rate with an increase in the conversion of glucose to lactic acid with the release of protons, causing a decrease in the pH level that leads to elevated acidity. This problem can be overcome by using chemical or biological agents targeting pH regulatory mechanisms. An alternative approach may be the encapsulation of CQ with proper nanoparticles for efficient delivery into cancer cells in the acidic tumor microenvironment. The second reason is that CQ, as an inhibitor of lysosomal autophagy, interferes with several functions of tissue turnover and with key events in the immune response.[33] This has been the subject of various studies, from which evidence is emerging that CQ can significantly strengthen the antineoplastic immune response, at least under certain conditions.[34] Analysis of related data shows that a substantial part of the effects of CQ is due to its interference with cellular metabolism, gene regulation, and mechanisms that trigger innate immunity including signaling pathways through Toll-like receptors (TLR) and tumor necrosis factor-α (see Supplementary material, Appendix S1).[35,36] It is important to note that cell stress from antineoplastic treatment and CQs can activate some transcription factors, which may reprogram the cells and thereby enable tumor cells to escape from cell death induced by drug treatment or from the immune system.[37,38] It is important to track the regulatory mechanisms at the molecular

Table 1. Clinical studies of chloroquines in cancer, registered with the database of the US National Library of Medicine at the National Institutes of Health. The safety and scientific validity of the listed studies has not been evaluated by the US Federal Government

Registration Identifier	Recruitment Status as of 11/2019	Aim	Responsible Party
224978	Completed	Chloroquine for Treatment of Glioblastoma Multiforme	National Institute of Neurology and Neurosurgery, Mexico
568880	Completed	Hydroxychloroquine and Bortezomib in Treating Patients With Relapsed or Refractory Multiple Myeloma	Abramson Cancer Center of the University of Pennsylvania
771056	Terminated	Hydroxychloroquine in Untreated B-CLL Patients	Northwell Health
969306	Terminated (Poor accrual)	Chloroquine as an Anti-Autophagy Drug in Stage IV Small Cell Lung Cancer Patients	Maastricht Radiation Oncology
1023477	Completed	Study of the Efficacy of Chloroquine in the Treatment of Ductal Carcinoma in Situ	Inova Health Care Services
1227135	Recruiting	Imatinib Mesylate With or Without Hydroxychloroquine in Treating Patients With Chronic Myeloid Leukemia	University of Glasgow
1396200	Completed	Cyclophosphamide and Pulse Dexamethasone With Rapamycin or Hydroxychloroquine for patients with relapsed/refractory multiple myeloma	Abramson Cancer Center of the University of Pennsylvania
1438177	Terminated	Chloroquine in Combination With VELCADE and Cyclophosphamide for Relapsed and Refractory Multiple Myeloma	NYU Langone Health
1446016	Completed	Chloroquine With Taxane Chemotherapy for Advanced or Metastatic Breast Cancer After Anthracycline Failure	The Methodist Hospital System
1469455	Completed	DNA Repair Inhibitor & Irradiation on Melanoma	DNA Therapeutics
1575782	Terminated (Poor accrual)	Chloroquine as an Anti-autophagic Radiosensitizing Drug in Stage I –III Small Cell Lung Cancer	Maastricht Radiation Oncology
1689987	Completed	Hydroxychloroquine, Cyclophosphamide, Dexamethasone, and Sirolimus in Treating Patients With Relapsed or Refractory Multiple Myeloma	OHSU Knight Cancer Institute
1727531	Completed	IDO2 Genetic Status Informs the Neoadjuvant Efficacy of Chloroquine in Brain Metastasis Radiotherapy	Main Line Health
1777477	Completed	Adjuvant Effect of Chloroquine on Gemcitabine	University of Zurich
1894633	Terminated	Study of Whole-brain Irradiation With Chloroquine for Brain Metastases	Instituto Nacional de Cancerologia de Mexico
2071537	Unknown	Chloroquine in Combination With Carboplatin/Gemcitabine in Advanced Solid Tumors	University of Cincinnati
2333890	Unknown	A Phase 2 Randomized, Double-blind Trial Evaluating the Effects of Chloroquine in Breast Cancer	Ottawa Hospital Research Institute
2366884	Recruiting	Clinical Evaluation of a New Form of Cancer Therapy Based on the Principles of Atavistic Metamorphosis	Dr. Frank Arguello Cancer Clinic
2378532	Recruiting	The Addition of Chloroquine to Chemoradiation for Glioblastoma	Maastricht Radiation Oncology
2432417	Not yet recruiting	The Addition of Chloroquine to Chemoradiation for Glioblastoma	Maastricht Radiation Oncology
2496741	Unknown	Metformin And Chloroquine in IDH1/2-mutated Solid Tumors	Universiteit van Amsterdam
2631252	Terminated (Inability to accrue)	Phase I Study of Mitoxantrone and Etoposide Combined With Hydroxychloroquine, for Relapsed Acute Myelogenous Leukemia	University of Pittsburgh
2786589	Recruiting	Plasmodium Immunotherapy for Lung Cancer	State Key Laboratory of Respiratory Disease
3243461	Recruiting	International Cooperative Phase III Trial of the HIT-HGG Study Group	University of Göttingen
3400865	Not yet recruiting	Cabergoline Combined Hydroxychloroquine/Chloroquine to Treat Resistant Prolactinomas	Ruijin Hospital
3979651	Not yet recruiting	MEK and Autophagy Inhibition in Metastatic/Locally Advanced, Unresectable Neuroblastoma RAS Melanoma	Hospices Civils de Lyon
4163107	Not yet recruiting	Combined Carfilzomib and Hydroxychloroquine in Patients With Relapsed/Refractory Multiple Myeloma	Norwegian University of Science and Technology

level and design drug combinations accordingly to prevent tumor cell recovery.

Several recent findings have led to the expectation that CQs will be incorporated in cancer treatment schemes. However, supporting experimental data will be needed to close the gap between clinical drug repurposing trials and the related innovative basic and preclinical research, targeting to delineate the mechanisms of how to incorporate the repurposed drugs into treatment combination protocols.[39,40] At high concentrations, CQs can interact with several types of macromolecules, causing some effects such as inhibition of drug sequestration and degradation.[41] However, due to the number and severity of characterized side effects of the systemic use of high CQ doses, it is imperative that translational study focuses on improving the antineoplastic effects of low micromolar drug concentrations.[6,42,43] To this end, nanotechnology is expected to help to decrease the systemic exposure to CQs and their derivatives.[44–46]

Although we will show that CQ has diverse effects on tissue metabolism and homeostasis in general, the key scientific field that has the potential to enable CQ incorporation in anticancer drug combinations is the study of interference with immune functions. In the next paragraphs, we will attempt to explain why we consider this field as essential to the effective use of CQ in treatment combinations.

CQ inhibits lysosomal acidification

Theoretically, if a chemical base is weak enough, it will react selectively with the most acidic component within a given system. CQ is a weak base[47] and the most significant effect of CQ as a cancer drug is its interference with lysosome acidification.[6] Mechanistically, when CQ enters the lysosome, it becomes protonated due to the high internal acidity. Accumulation of protonated CQ in the lysosome increases lysosomal pH and consequently decreases lysosomal function.[48] However, a recent study proposed that CQ can also inhibit autophagy by impairing autophagosome-to-lysosome fusion,[49] an important observation that needs further investigation.

Some cell types rely on lysosomal degradation of macromolecules, especially when they depend on mitochondrial oxidative phosphorylation to generate energy. Damaged mitochondrial proteins are then gradually degraded in lysosomes.[50] Likewise, various other conditions such as drug-induced stress or mutations in the mitochondrial DNA may initiate mitochondrial degradation. Therefore, the cells under metabolic stress can target damaged mitochondria to lysosomes.[51,52] Moreover, some cancer cell types exhibit excessive acidity in their lysosomes, reaching pH values <4.[53] In addition, mediators of apoptotic cell death can also be degraded in lysosomes, a process that gives a survival advantage to cancer cells.[54,55] CQ may also lead to a decrease in the quality and functions of mitochondria.[56,57] Therefore, the inhibitory effect of CQ on mitochondrial function and lysosomal acidification can be explored for a synergistic therapeutic benefit.

Nevertheless, degradation of intracellular components is not the only role of lysosomes. For example, macrophages and dendritic cells use lysosomes for microbe killing and antigen processing and presentation.[58] Yet, another important role of lysosomes is the removal of dead cell debris to enable resolution of inflammation and tissue remodeling.[59] Scavenging M2-type macrophages are particularly effective in removing debris from tissues to help resolve inflammation; M2 macrophages have a highly acidic lysosomal pH (<5), whereas M1 macrophages that stimulate immune responses show lower lysosomal acidity (pH >5). An M2-to-M1 macrophage transition could be experimentally achieved by CQ at least partially through raising lysosomal pH.[60] Moreover, CQ has the potential to alleviate pathological conditions associated with increased M2 activity, such as vascular disorder during lung carcinogenesis.[61] Interestingly, it has also been shown that CQ attenuates lipopolysaccharide-induced M1 macrophage activation.[62] Dendritic cells also limit lysosomal acidification to optimize antigen processing and allow major histocompatibility complex class I (MHC-I) dependent cross-presentation.[63] CQ may facilitate antigen cross-presentation through late endosomes, and inhibit presentation from early endosomes, which is consistent with the inhibition of lysosomal acidification.[64,65]

CQ is an inhibitor of late autophagy

In eukaryotic cells, autophagy is an evolutionarily conserved mechanism that plays important roles in degradation and recycling of intracellular components.[66] This mechanism is a multi-step complex process and involves the engulfment of targets by autophagosomes and their subsequent degradation by lysosomes.[66,67] Lysosomal degradation is the last step in the process of autophagy, which generates molecules to be used for the synthesis of macromolecules.[67]

Autophagy allows cells to degrade long-lived proteins, aggregates and entire organelles.[68] Processes of autophagy include mitophagy (for mitochondria), pexophagy (for peroxisomes), ER-phagy (for endoplasmic reticulum), or ribophagy (for ribosomes), depending on which organelles are targeted for specific autophagic degradation.[69] Hence, autophagy has the capacity to influence the entire spectrum of macromolecular turnover in a tissue. Under certain conditions, cells may undergo autophagic death, if intracellular survival pathways are blocked during autophagy.[15,70,71] Pharmacological inhibitors of autophagy have been used extensively in various cellular conditions to delineate the molecular mechanism of autophagy and the

effect of these inhibitors on autophagy. CQ is one such pharmacological inhibitor of autophagy that has been extensively used in research. It inhibits late stages of autophagy and may induce cell death even under conditions where inhibitors of early autophagy cannot[72] (Fig. 1b). Furthermore, it impairs the late stages of acidic organelle fusion, especially autophagosome–lysosome fusion.[49]

Autophagy regulates cell and tissue homeostasis

Senescence is an important homeostatic process. It is triggered by various cell stress factors and arrests proliferation, which may lead to tumor cell survival and limit the therapeutic effects of pharmaceutical agents. Depending on the substrates targeted to the lysosomes, autophagy can prevent or accelerate cellular senescence.[73] Specifically autophagy suppresses cellular senescence by removing damaged macromolecules or organelles, or promotes cellular senescence by facilitating the synthesis of senescence-associated secretory proteins.[73] This dual capacity of autophagy can therefore enable a tissue to either rescue or remove cells with malfunctioning organelles. This would preserve the integrity of tissue structure and function. At the organismal level, aging impairs regulation of autophagy, which may characteristically lead to a decreased protection of the heart, for example, from ischemia–reperfusion injury.[74] For instance, fasting induces transcription factor EB (TFEB) which activates mitochondrial and lysosomal biogenesis, and enables autophagy, to allow removal and replacement of damaged organelles. TFEB protects the heart from cardiac injury during ischemia–reperfusion, which would otherwise cause cell death by hypoxia–reoxygenation.[75] Drugs that interfere with the activation of TFEB, such as anthracyclines, impair autophagy and cause cardiomyopathy.[76] The interference of anthracyclines with TFEB activity can also impair autophagy of peroxisomes, pexophagy, which may contribute to neurotoxicity, cognitive dysfunction, and accelerated brain aging in cancer patients and survivors.[77]

Autophagy has crucial roles in vertebrate development from the pre-implantation stage to organogenesis.[78,79] Abnormalities in autophagy may lead to various developmental defects, as shown in several model organisms.[79] For example, during chick embryogenesis, autophagy is essential in regulating the temporal expression and spatial localization of developmental genes and in coordinating epithelial-to-mesenchymal transitions that enable the establishment of the three germ layers.[80] Consistently, developmental delay has been observed in chick embryos when autophagy was inhibited.[80]

CQ regulates tissue responses to metabolic and inflammatory stimuli

A tissue may regularly need cells to undergo autophagy as a means to remove damaged or misfolded proteins, conserve resources, and restore homeostasis. This is evident in a few experimental systems, where CQ exacerbates metabolite imbalance and causes tissue dysfunction and damage. For example, low micromolar CQ can exacerbate vascular calcification.[81] Likewise, autophagy protects cardiomyocytes in mice fed a high-fat diet, and this beneficial effect can be abrogated by CQ.[82] However, HCQ may protect the myocardium in combination with phosphodiesterase inhibitors in type 2 diabetes.[83] Autophagy in the heart is enhanced in type 1 diabetes but is suppressed in type 2 (insulin-resistant) diabetes.[84] Therefore CQ, being an inhibitor of late autophagy, might exacerbate cardiomyopathy in type 1 diabetes by causing myocardial hypertrophy and interstitial fibrosis.[85] Also, in type 2 diabetes, inhibition of autophagy by CQ can be detrimental for circulation, especially in endothelial progenitor cells that are needed to preserve ischemic angiogenesis and blood perfusion.[86]

Autophagy is not always beneficial though; in excess, it can contribute to tissue damage, both in young and aged mammals.[87,88] Even in bone marrow, where autophagy provides essential maintenance function, excessive autophagy of mesenchymal stem cells is very likely the cause of cellular senescence during hyperglycemia-induced marrow hematopoietic niche dysfunction.[89] Treatment with the rapamycin can induce non-coding RNA LCPAT1 and activate autophagy, to permit growth of lung tumors.[90] It is interesting to note that increased lysosomal degradation can even suppress nuclear receptor signaling and can thereby blunt the glucocorticoid-mediated inhibition of gene expression of inflammatory cytokines. Hence, CQ has the potential to modulate inflammation, in synergy with glucocorticoids.[91] Viral infection, especially Epstein–Barr virus, can activate constitutive autophagy to support virus latency and permit lymphoproliferative disorders and lymphomas.[92] Epstein–Barr virus-induced autophagy is dependent on the expression of viral latent membrane proteins, which provide cells with an improved survival ability. In these cells, viruses inhibit lysosomal degradation in the maturation step of autophagy and use autophagic membranes for the formation and release of the viral particles. Therefore, inhibiting lysosomal degradation with CQ induces p53-dependent cell death and prevents cancer in mouse models of lymphomagenesis.[93] In fact, basal autophagy has been proposed to play a pivotal role in sustaining mitochondrial function in lymphoma, and low CQ concentration may cause apoptosis in susceptible lymphoma cells.[94]

In contrast, inhibition of autophagy by CQ may exacerbate diabetic neuropathy and impair neuronal function.[95] Lysosomal proteolysis enables mitochondrial quality control in rat hippocampus, a process impaired by CQ.[96] Likewise, the activity of autophagy is closely related to muscle diseases and inhibition of autophagy by HCQ may cause severe vacuolar myopathies in patients with

Danon disease.[97] CQ can also affect tissues such as kidney and pancreas. Similarly, CQ (10 mg/kg/day) can exacerbate renal ischemia/reperfusion injury in type 2 diabetes.[98] In type 2 diabetes, low micromolar CQ could inhibit autophagy and cause apoptosis of podocytes and exacerbate nephropathy.[99] In the pancreas of individuals with type 2 diabetes, cytokine interleukin-6 (IL-6) induces autophagy and protects pancreatic beta cells from apoptosis whereas CQ causes apoptosis and thereby elicits tissue damage.[100]

Failure of progenitor cells to regulate autophagy during tissue regeneration can contribute to carcinogenesis.[37,68] Likewise, cells can activate autophagic degradation of proteins as a compensatory mechanism under drug-induced cytotoxic stress.[101,102] Through activation of cellular protein recycling during autophagy, lysosomes can help a cell survive endoplasmic reticulum stress that is often caused by anticancer drugs.[37,103] Endoplasmic reticulum stress otherwise kills the cell by inducing the unfolded protein response, mitochondrial membrane depolarization, and ensuing activation of caspases and degradation of DNA.[104] Hence, autophagy could function as a rescue mechanism for malignant cells against treatment-induced cell stress. In cancer, however, malignant cells might also acquire the capacity to induce autophagy of the mesenchymal cells in tumor stroma, to supply nutrients and generate a niche that fosters cancer cell survival and proliferation.[32,105]

Chloroquine can also synergize with agents targeting the cAMP pathway, such as adenosine monophosphate-activated protein kinase (AMPK). For example, in response to ATP deficiency, AMPK is activated and induces autophagy through inhibition of the mammalian target of rapamycin.[106] AMPK activation thereby facilitates the restoration of the cellular energy status by switching on a catabolic pathway to generate ATP while simultaneously inhibiting ATP-consuming processes such as cell proliferation and biosynthesis. However, when AMPK is aberrantly activated it can cause severe pathological manifestations associated with excessive glycolysis.[107] Induction of autophagy by AMPK causes cell death when combined with inhibition of late autophagy by CQ.[108] In some cases, the combination of CQ and AMPK activators, such as acetaminophen, can lead to side effects, such as liver toxicity.[109] CQ-AMPK synergy can also lead to cancer cell death.[110] Examples include synergy of CQ with the two AMPK activators, the glucose analog 2-deoxyglucose in killing prostate cancer cells, and with OSU-53 in killing triple-negative breast cancer cells.[111,112] Both of these AMPK activators also inhibit activation of transcription factor signal transducer and activator of transcription 3 (STAT3).[111,113] STAT3 can activate the expression of proteasome subunits such as β subunits of the 20 S proteasome core complex and immunoproteasome subunits latent membrane proteins7 and 2.[114,115] Thereby, STAT3 regulates proteostasis through the proteasome, a module that interacts with autophagy as we see next.[116] STAT3 itself is a therapeutic target in cancer, and at least in some study systems, STAT3 blockers can be combined with autophagy inhibitors. In cancer cells, tyrosine kinase inhibitors can block STAT3 signaling, and thereby activate autophagy, making cells sensitive to death by CQ treatment.[117–119] Hence, CQ regulates proteostasis, proteasome activity, and cell viability.

Proteostasis regulates cellular stress responses

In particular the Ubiquitin–Proteasome system (UPS) is a cellular mechanism degrading proteins that complements the activity of the lysosome.[116] In general, proteins with a short half-life undergo programmed degradation in the UPS after having completed their function.[120] In addition, soluble misfolded and unfolded proteins can also be degraded by UPS.[121] UPS is involved in vital cellular processes such as regulation of cell cycle progression, transcription, and DNA repair.[122–124] The activities of UPS and autophagy are linked, and inhibition of the one causes activation of the other.[116] Inhibitors of the proteasome and several anti-inflammatory agents cause the redistribution of targeted proteins in organelles.[125] Some protein aggregates inhibit proteasome function but trigger lysosomal protein degradation through a number of mechanisms.[116,126] The inhibition of proteasome induces transcription of p62 via transcription factor nuclear factor erythroid-related factor 1 (NRF1).[127] p62, also known as Sequestosome 1 (SQSTM1), is a ubiquitin-binding adaptor protein that bridges the proteasome-dependent degradation process to autophagy.[128] It is a multifunctional protein, and its different domains are involved in both UPS and autophagy-dependent degradation processes.[128] Proteasome inhibition triggers autophagy by increased endoplasmic reticulum stress that releases NRF2 from Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1, leading to expression of NRF2 target genes that induce autophagy.[129] Also, the transcription factor early growth response protein-1 is a substrate of the proteasome and activates expression of genes within the autophagy pathway.[130,131] Conversely, RING (really interesting new gene)-domain ubiquitin E3 ligases, which target proteins for proteasomal degradation, regulate autophagy and are themselves degraded by autophagy.[132]

The cellular proteolytic systems are therefore regulated in a coordinated fashion to enable adequate distribution of molecular resources according to changes in growth conditions. Practically this means that inhibition of one proteolytic system activates another proteolytic system. Inhibition of UPS by chemical agents leads to the activation of autophagy by increasing the expression levels of several autophagy-related genes.[133,134] Consistently, the

activity of UPS was increased when autophagy was inhibited by chemical agents or by small interfering RNAs targeting autophagy-related genes.[135,136]

There are several examples of this complementarity. When proteasome activity is impaired, its substrates may be imported into mitochondria to be degraded by mitophagy.[137] Transcription factor NF-κB is induced by inflammatory stimuli that activate proteasomal degradation of inhibitor of NF-κBα (IκBα), which otherwise inhibits NF-κB by sequestering it in the cytoplasm.[138] After the degradation of IκBα, NF-κB is translocated into the nucleus and interacts with nuclear hormone receptors, stress mediators, and tumor suppressors, to activate the expression of genes with different expression dynamics.[139,140] DNA damaging agents increase the activity of NF-κB, both by increased recruitment to chromatin, as well as by increased interaction with protein complexes that recruit RNA polymerase II.[141–143] As a mechanism of negative feedback regulation, the IκBα gene is expressed by activated NF-κB.[144,145] To sustain NF-κB activity in some cells, inhibition of the proteasome may induce proteolysis of IκBα by the lysosome, and inhibition of the lysosome can induce proteolysis of IκBα by calpain.[146,147] The degree of redundancy of these proteolytic systems in IκBα degradation depends on the cell type and the phase of the inflammatory cascade.[148–150] In endothelial cells, inflammatory cytokines induce degradation of IκBα by autophagy, which leads to the expression of vascular cell adhesion molecule 1.[151] By activating expression of vascular cell adhesion molecule 1, autophagy then enables the next step in the inflammatory cascade, which is the adhesion of lymphocytes to the endothelium and the recruitment of immune cells to the inflammation site.[152]

With regard to leukocytes, it was shown that 10 μM CQ caused M1 macrophage polarization through lysosomal calcium release and activation of protein kinase p38 and NF-κB.[60] Similarly, the proteasome inhibitor bortezomib induced autophagic degradation of IκBα leading to the activation of NF-κB in diffuse large B-cell lymphoma cells.[153] Specifically, p62 recruits ubiquitinated proteins, including IκBα, to autophagosomes. Consequently, NF-κB activation and degradation of p62 and IκBα was blocked by a higher CQ concentration (50 μM), which potentiated lymphoma cell death by bortezomib (Velcade). Furthermore, p62 can activate NF-κB through diverse pathways.[154] Autophagy can also activate NF-κB by sequestering its inhibitor A20, thereby permitting expression of chemokines CXCL1 and CXCL2, recruiting neutrophils, and extending antimicrobial inflammatory responses.[155] This partial redundancy may allow cell survival under inflammatory or toxic insult conditions.[37] Under conditions of the physiological activity of the UPS, IκBα proteolysis permits NF-κB to activate IκBα re-synthesis, which in turn inhibits NF-κB activity by sequestering NF-κB in the cytoplasm. If NF-κB was the only factor capable of inducing IκBα expression, then NF-κB activation would function as a self-limiting cycle, whereby NF-κB activity gradually returned to basal levels.[156]

Expression of cell stress regulators affects cancer prognosis

In contrast to inflammatory genes, some endogenous anti-inflammatory mediators, such as glucocorticoids, can activate IκBα and thereby maintain the inflammatory genes in a repressed state under normal homeostatic conditions.[157] However, in triple-negative breast cancer cells, the recognition of substrates by IκBα is dysregulated and therefore IκBα is not sufficient to block expression of NF-κB-driven inflammatory genes.[158,159] The expressed inflammatory genes inactivate natural killer (NK) cells and recruit neutrophils into both primary tumor and lung pre-metastatic niche, enabling breast cancer cell metastasis.[160] Can there be a prognostic impact of this dichotomy between triple-negative breast cancer and the other breast cancer types with more efficient NF-κB regulation by IκBα?

The fact that IκBα gene expression becomes less relevant in triple-negative breast cancer cells can be illustrated by contrasting its effects on prognosis, when compared with the other types of breast cancer. In the case of triple-negative breast cancer, neither higher IκBα expression nor a higher ratio of IκBα/ p62 gene expression offers survival benefit as seen in The Cancer Genome Atlas database (http://cancergenome.nih.gov/), whereas in other types of breast cancer, both higher IκBα as well as a higher ratio of IκBα/p62 gene expression has a significant positive effect on prognosis (Fig. 2a). The impact of changes in expression was calculated using the database PROGGENEV2.[161] Even higher prognostic divergence between triple-negative and other types of breast cancers can be shown with the ratio between IκBα and Autophagy related 4C cysteine peptidase (ATG4C), which is required for stress-induced autophagy, (such as under conditions of oxidative stress or prolonged starvation)[162,163] (Fig. 2b). Conversely, an eight-gene signature composed of four autophagy-related and four proteasome-related genes offers a survival advantage in only triple-negative breast cancer, while lacking this effect in the other types of breast cancer (Fig. 2c). This clearly shows a divergence in proteostatic effects for triple-negative breast cancer and is in agreement with the hypothesis of a decreased impact of IκBα levels.

The dichotomy in prognostic effects between ATG4C and IκBα is remarkable and shows that while a proper autophagic stress response protects from cancer, overexpression of a key component of this response mechanism in disproportion to the expression of anti-inflammatory protein IκBα is tightly linked to negative prognosis. Furthermore, while both p62 and IκBα are targets of CQ,

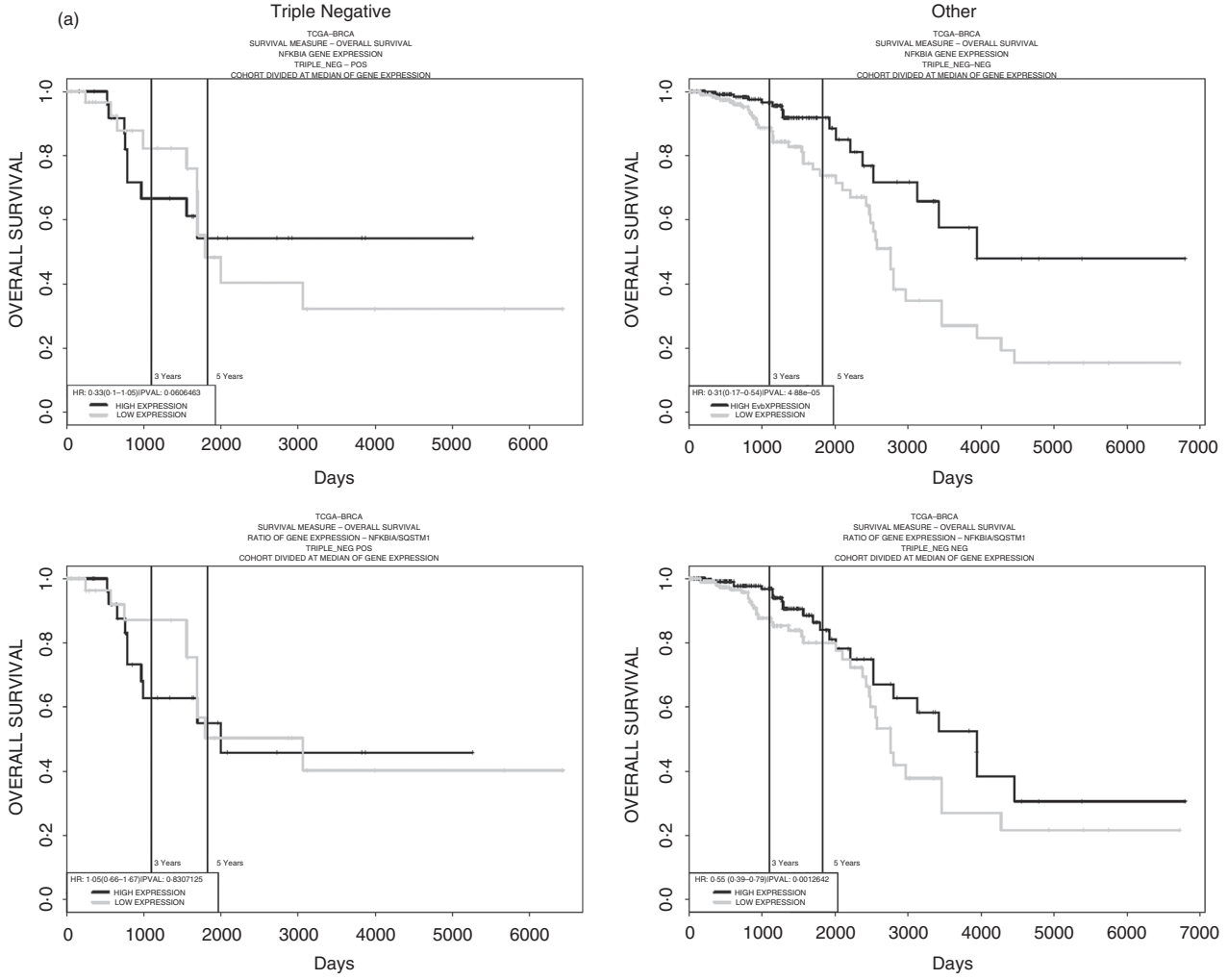

Figure 2. Inhibitor of NF-κBα (IκBα) is a key mediator at the interface between autophagy and the proteasome, and therefore the effect of its overexpression on cancer prognosis is an important factor. In triple-negative breast cancer, the overexpression of proteasome and autophagic components (their gene products are also involved in IκBα degradation) is a positive prognostic factor, while in non-triple-negative breast cancer, it is IκBα that becomes a positive prognostic factor. This highlights the importance of proteostasis (proteasome and autophagy) in cancer prognosis. (a) Prognosis in triple-negative breast cancer is not affected by expression of the gene encoding IκBα (*NFkBIA*) nor is prognosis affected by the ratio between the expression of *NFkBIA* and the p62 adaptor protein Sequestosome 1 (*SQSTM1*). In contrast, in other types of breast cancer, this ratio has a significant impact on survival. (b) The same divergence in prognosis between triple-negative and the other types of breast cancer is true for the ratio between IκBα and ATG4C. (c) An eight-gene signature (*PSMD11*, *PSMG3*, *PSMB4*, *PSMC3*, *ATG4C*, *UVRAG*, *MAP1LC3A*, *MAP1LC3C*) correlates with a survival advantage only in triple-negative breast cancer (Black curve: high expression; grey: low expression)

their expression has divergent results depending on the cancer subtype. As IκBα gene expression is a point of convergence of negative feedback mechanisms of inflammation, the dichotomy in prognostic effects demonstrates the importance of proteostasis in the regulation of genes that affect tissue integrity.

Cell stress regulators are partially redundant: impact on cancer progression

Nevertheless, NF-κB activity may be inhibited at different levels; for example by marking of the upstream NF-κB activating proteins tumor necrosis factor receptor-associated factor 2 (TRAF2) and TRAF5 for degradation by the lysosome.[164] This is important in the regulation of cell fate, as autophagic degradation of TRAF2 limits the capacity of a cell to undergo NF-κB-dependent epithelial to mesenchymal transitions.[164] NF-κB itself regulates genes that encode proteins operating both inside the cell, in its organelles, and in the extracellular space, and thereby controls all cellular functions, including internal homeostasis and communication with the microenvironment[38,147] (Fig. 3). Furthermore, NF-κB also regulates the expression of autophagy genes to enable cellular adaptation and survival under adverse conditions.[165]

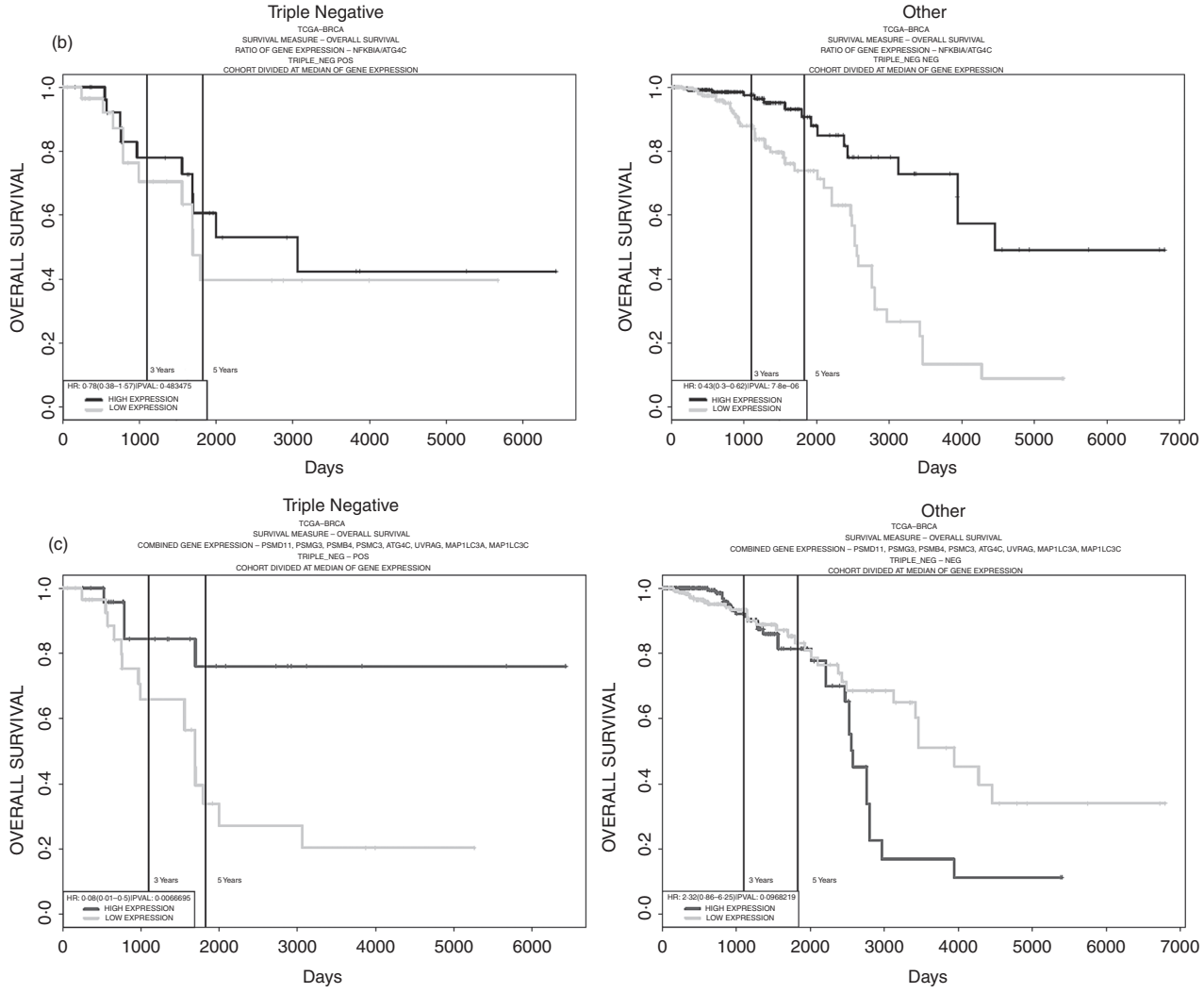

Figure 2. Continued

In cancer cells, adaptation mediated by the autophagy–proteostatic feedback is detrimental for the host, whereas in normal cells, this mechanism preserves essential tissue homeostasis. In melanoma cells, 25 μM CQ suffices to cause accumulation of the autophagy adaptor protein p62, inducing NF-κB activity and thereby leading to expression of survival genes and p62 itself, culminating in cell resistance to apoptosis.[166] Nevertheless, in normal cells, coordinated proteostatic feedback is beneficial for the host tissue. An example of such a beneficial function of the proteostatic feedback is seen when NF-κB promotes mitophagy through induction of p62 expression in macrophages, thereby preventing the accumulation of damaged mitochondria and limiting excessive IL-1β-dependent inflammation.[167]

In the opposite direction, mechanisms that induce inflammation limit autophagy. NF-κB-target gene IL-6 limits autophagy in healthy tissues.[100] One can then ask, how do cells reprogram expression from inflammatory genes to autophagy genes if both share some of their transcriptional activators? The answer probably lies in the recruitment of chromatin regulators, whose activities are regulated through metabolic functions. Bromodomain and Extra Terminal domain (BET) family proteins are epigenetic readers that recognize histone acetylation and promote expression of inflammatory genes, whereas histone deacetylases such as sirtuin 1 (SIRT1) function as epigenetic erasers and promote expression of autophagy genes.[168,169] BET functions as a repressor for some genes such as TFEB, whereas activated AMPK allows SIRT1 to displace BET.[170] This function could be expected to promote organelle restoration and cell survival under conditions of depleted nutrients and oxidative stress during inflammation. Indeed, negative feedback between autophagy and inflammation protects tissue integrity in the normal homeostatic state but fails in pathological states

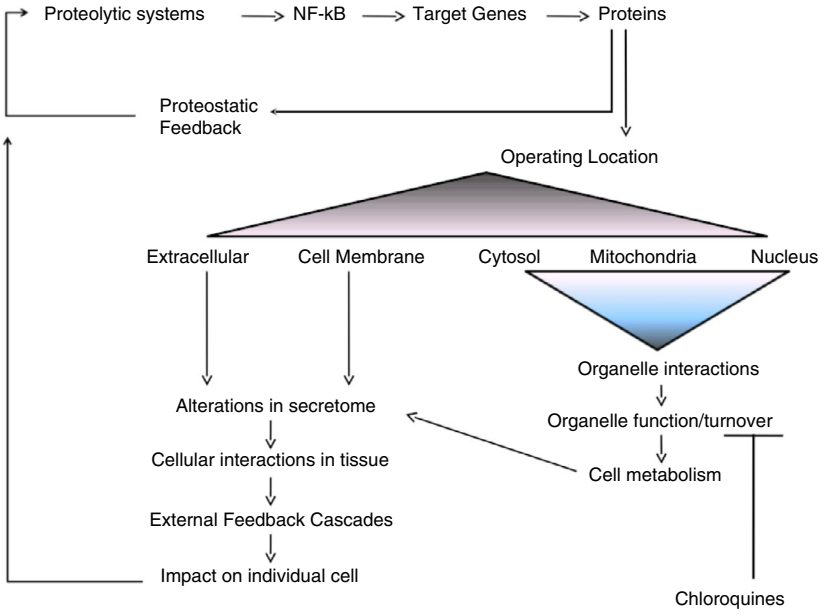

Figure 3. Proteostasis regulates gene expression and thereby controls the extent and duration of inflammation and cell stress. As an example, the transcription factor nuclear factor-κB (NF-κB) is induced by inflammatory stimuli and controls the expression of intracellular proteins and extracellular mediators. Proteolytic systems, primarily the proteasome and secondarily other systems such as lysosome, degrade the immediate NF-κB inhibitor nhibitor of NF-κBα (IκBα) to facilitate the pro-inflammatory activity of NF-κB, and expression of cell survival genes. Normally regulation of gene expression by NF-κB ultimately includes cessation of transcriptional activity. A number of negative feedback mechanisms resolve inflammation by inhibiting pro-inflammatory NF-κB activity. Inhibition of lysosomal acidification by CQs disrupts the regulation of proteostasis, and a significant part of the linked molecular turnover.

such as acute lung injury.[171–173] Hence, intracellular IκBα proteostasis controls the immune system and tissue homeostasis through relocation of NF-κB.

Disruption of tissue capacity to regulate autophagy can generate a suitable niche for senescent stromal cells that can either enhance tumor growth or provide a niche for metastatic cancer cells.[174,175] In fact, cancer cells may actively reprogram autophagy in stromal cells to promote cancer progression.[176,177] On the other hand, cancer cells treated with antimitotic drugs may escape mitotic arrest by entering interphase without proper chromosome segregation. This escape is linked to the induction of autophagy and leads to cellular senescence, migration, invasion and vascularization.[178] Therefore, both impaired autophagy and excessive or dysregulated autophagy can disrupt tissue homeostasis and favor cancer progression (Fig. 4).

CQ effects on cancer stem cells

Although conventional cancer treatment approaches reduce tumor mass/volume, they often fail to eradicate all tumor cells.[179] The major reason for treatment failure is related to tumor heterogeneity.[180] It has been proposed that cancer stem cells (CSC) or cancer-initiating cells (CIC) with self-renewal potential may be responsible for the cell heterogeneity in tumors.[181] CSC or CIC are small sub-populations of the cells that give rise to various cancer cell types and have the capacity of self-regeneration within a tumor.[182]

Several properties of CSC affect the impact of CQ on tumors. CSC may derive from cells in a variety of differentiation stages, and autophagy bestows them with a critical degree of metabolic plasticity.[183] During tumor growth, rapidly dividing cells that rely on glycolysis produce lactate. This, in turn, generates an acidic microenvironment that when combined with hypoxia leads to the development of quiescent cancer cell clones that share properties with stem cells.[184] These quiescent cells rely on autophagy.[185] Through autophagy, cancer cells and especially cancer stem-like cell clones increase their capacity for material turnover and efflux of cancer drugs.[186,187] Therefore, autophagy has been considered as a major factor for the survival and chemotherapy resistance of CSCs.[188] These protective effects of autophagy on CSC have been consistently shown in various cancers including colon and breast cancers and chronic myeloid leukemia.[189–191] In addition, autophagy was proposed to enable neoplastic cells that have undergone epithelial-to-mesenchymal transitions to acquire CSC traits.[192] Moreover, CSC markers and autophagy have been associated with poor prognosis in pancreatic cancer patients.[193] Therefore, inhibition of autophagy is a promising approach to overcome chemotherapy resistance and consequently to eradicate tumor cells by reducing tumor heterogeneity.

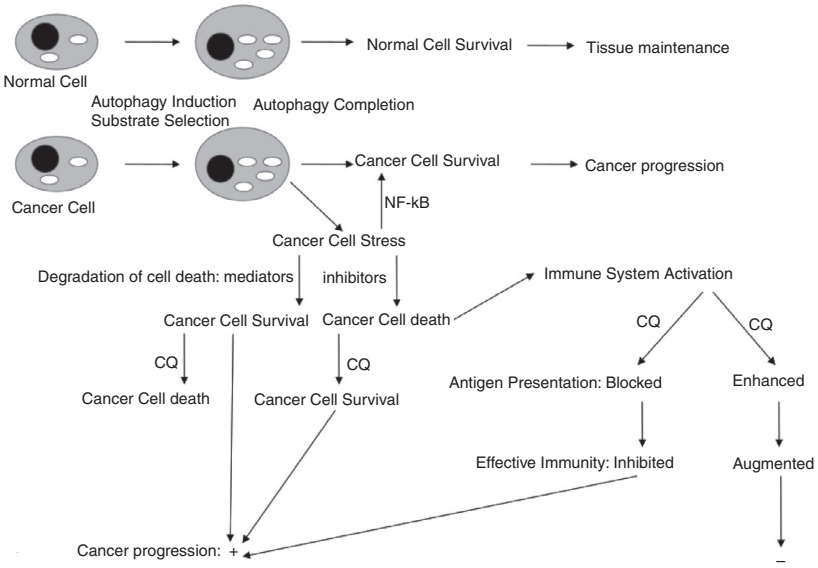

Figure 4. Chloroquines are expected to have diverse effects on cancer progression. Inhibition of late autophagy can affect cell survival, as well as antigen processing and presentation in the immune system. Combinatorial study of these effects at several levels will help enhance the capability to incorporate them in cancer treatment

In general, the capacity of CSC to maintain their carcinogenic potential is linked to their cell stress response mechanisms. As an example, CSC can increase their DNA double-strand breaks, and thereafter induce the enzymatic activity of the ataxia telangiectasia mutated kinase (ATM), leading to activation of transcription factors NF-κB and STAT3,[194] two important transcription factors involved in tumorigenesis.[195] Their deregulated activities were shown in various cancers including liver, lung, and prostate.[196] In addition, STAT3 induces a number of DNA repair mechanisms, including ATM itself.[197] ATM can further be induced by diverse conditions involving cell stress and organelle dysfunction.[163] Moreover, alkylating agents such as temozolomide activate ATM, which induces cytoprotective autophagy to rescue tumor cells from chemotherapy, and even rescue cells from inhibition of NF-κB and STAT3.[198]

Twenty micromolar CQ interferes specifically with ATM-induced autophagy and stem cell-like features in CSC.[185] In a reported patient case with a follow up of 24 months, 150 mg/day CQ was well tolerated within an extensive ketogenic cancer treatment scheme that, in addition to temozolomide, also included AMPK activator metformin at 1 g/day.[199] A clinical trial was initiated to determine metformin/CQ maximum tolerated doses for the treatment of solid tumors.[200] Similar to other AMPK activators, mentioned herein, metformin inhibits activation of STAT3.[201] Hence, it is becoming increasingly possible to evaluate the combination of autophagy activators with late-stage autophagy inhibitors in the clinics.

The low micromolar concentration of CQ (1 or 5 μM) shows a striking capacity to decrease the expression of DNMT1, an important DNA methylation regulator that is highly expressed in various cancers and interferes with Janus-activated kinase 2—STAT3 signaling in breast cancer CSC.[202] In solid tumors, CQ interferes with STAT3, Hedgehog and CXCR4 signaling, as well as with autophagy in CSCs that are identified by the markers aldehyde dehydrogenase, CD44, and CD133.[6,203] In fact, aldehyde dehydrogenase sensitizes cancer cells to lysosomal autophagy inhibitors, including HCQ. Expression of helicase-like transcription factor, a member of switch/sucrose non-fermentable family proteins that regulate chromatin/nucleosome remodeling, promotes DNA damage repair and HCQ resistance, which indicates that HCQ could be combined with DNA repair inhibitors.[204]

Another class of inhibitors with which HCQ and CQ can be combined, are the inhibitors of the BET epigenetic readers, which drive the expression of genes promoting inflammation and cell survival.[38,170] What is encouraging is that a relatively low CQ/HCQ concentration (10 μM), in synergy with BET inhibition, is sufficient to cause apoptosis in CSC of both pancreatic cancer and acute myeloid leukemia.[170,205] CQ can also kill cancer cells in combination with cell cycle inhibitors.[206,207] A combination of CQ and proteasome inhibitor causes apoptosis in human liver tumors orthotopically or subcutaneously xenografted in mice.[208] This demonstrates the importance of proteostasis for tumor cells.

Chloroquine and other inhibitors of autophagy have cytotoxic effects on diverse leukemia-initiating cell types such as CD34-positive and glucocorticoid-resistant clones.[205,209] However, in leukemia patients, high doses of CQ are needed to inhibit autophagy, which limits their

therapeutic efficacy.[210] One potential explanation comes from the observation that CQ may kill leukemia cells independently from the early steps of the autophagy pathway, and this CQ effect can be neutralized by leukemia cells *in vivo* through exocytosis.[211]

There exists yet another mechanism that impairs the antineoplastic activity of CQ in a patients' tissue. Cancer cells commonly cause a localized acidosis that prevents the entry of CQ.[30,212] Nevertheless, CQ could still reach migrating cancer cells that have not yet entered or established a sufficiently acidic tumor niche to block CQ entry.[213] Such a niche results from interactions between tumor and stromal cells.[214] Hence, CQ can affect metastatic cells in transition and this can explain the statistically significant effect of CQ against metastatic cancer cells in patients.[215] Furthermore, metastatic cells are preferentially vulnerable to lysosomal inhibition.[216] The capacity of CQ to affect cancer cells in transition can also be linked to the interactions between transcription factors that steer cellular metabolism.[217,218] As an example, interactions between NF-κB, STAT3, p53, and the glucocorticoid receptor (GR) guide dynamic transitions in the structures and functions of a cells' organelles and the resulting changes in the cells' phenotype.[219–222] Organelles, in turn, form intracellular networks that control autophagy and cell death.[223,224]

Transcription factors such as NF-κB, STAT3, p53, and GR interact at various levels. Their interactions impact the progress of the cell cycle, the response to nutritional changes, the induction of cell stress, organelle function, recycling and expression of cell surface molecules, and the secretion of diverse chemo-attractants.[225–228] The importance of NF-κB, STAT3, p53, and GR interactions is especially pronounced in malignant cells, and guides the cells' interactions with host tissue.[147] CQ also impacts the dynamic interface between NF-κB, STAT3, p53, and GR, and the phenomena these proteins control, including mitochondrial membrane potential[229] and mitophagy.[230] Although this interference can be associated with undesirable side effects of pharmacological CQ use at higher concentrations, it also forms a basis for the treatment of malignant tumors, especially to target drug-resistant CSC.

Impact of CQ on antitumor immune responses

Antitumor immune response requires autophagy. Autophagy enhances immunogenic cell death and tumor antigen processing and presentation, thereby promoting adaptive antitumor immunity.[231] In tumor cells, autophagy leads to immunogenic cell death by surface exposure of calreticulin (ecto-CRT), secretion of ATP, and release of apoptotic proteins such as HMGB1. In antigen-presenting cells, autophagy promotes antigen presentation by MHC.[34] Lysosomal acidification in distinct phases of the pathways that lead to antigen presentation is needed and this need depends heavily on the nature of the antigen.[64] MHC-I-driven presentation, which tends to be promoted by CQ, exposes cancer-derived neoantigens to the immune system.[232,233]

On the other hand, autophagy can also suppress immune responses. Autophagy, especially under hypoxia, permits activation of transcription factor STAT3, leading to the expression and secretion of cytokines such as IL-10, and a number of other factors with immunosuppressive effects that help cancer cells escape multiple components of the immune system.[34] This phenotype has often been observed in solid neoplasms and generally leads to more aggressive treatment-resistant tumor phenotypes.[9] Consequently, blocking the hypoxia-induced autophagy in tumors restored cytotoxic T-cell activity and promoted regression of melanoma xenografts in mice.[234] Clearly, cancer is a complex condition, and difficult to describe immunologically. Therefore, the analysis of immune responses to antigens related to other diseases can aid in defining the immunological effect of CQs.

Do CQs have an immunosuppressive effect, especially at the antigen presentation level? In the case of malaria, an effective T helper type 1 (Th1) -dominated immune response requires TLR4, TLR9, interferon-γ (IFN-γ), and immunoglobulins IgG2a, IgG2b, and IgG3.[235,236] In malaria, a critical 3-day time course is needed to mount an immune response before a 50 mg/kg dose of CQ can be added to the treatment regimen.[237]

The capacity of CQ to protect tumors from the mammalian immune system is evident by adding CQ to curcumin in immunocompetent mice. Namely, CQ treatment increases the cytotoxic effect of curcumin against epidermal growth factor receptor 2-overexpressing breast cancer cells in nude mice while counteracting it in immune competent mice.[238] Moreover, it was shown that CQ inhibits acidification of endolysosomes and consequently impairs antigen presentation to $CD4^+$ T cells in HIV infection.[239] Similarly, it was reported that HCQ inhibits intracellular TLR signaling in rheumatoid arthritis.[239]

Chloroquine has a beneficial immunosuppressive effect against IFN-γ-directed immune responses in autoimmunity and especially in lupus, where it balances the effect of autophagy in Th17 and regulatory T (Treg) cells, reducing IFN-γ and inflammatory cytokines.[240] Lupus also involves TLR9, IgG2a, and IgG2b, consistent with the beneficial immunosuppressive effect of CQ.[241] In lectin-stimulated human peripheral blood mononuclear cells, CQ inhibits soluble IL-2R expression dose-dependently at concentrations between 10 and 50 μM. It is interesting that under the same conditions, 10 μM CQ augments, while 50 μM CQ blocks neopterin expression, which suggests a potential of CQ to normalize IFN-γ signaling in some tissues.[242] Another potential beneficial effect of CQ against autoimmunity is evident in experimental

autoimmune encephalomyelitis, a mouse model for multiple sclerosis. This condition is ameliorated by a CQ treatment protocol (5 mg/kg/day for 5 consecutive days) that promotes the expansion of Treg cells and decreases inflammatory cells.[243] CQ-treated mice showed a significant reduction in the number of IL-17A- and IFN-γ-producing cells and a significant increase of IL-10-producing cells in the central nervous system.

On the other hand, CQs show a capacity to stimulate immune responses under certain conditions. T-cell-deficient mice depend on the activity of NK cells and on the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) signaling, to mount antitumor immune responses.[244] However, tumor cells may escape TRAIL and NK cells by activating autophagy.[245,246] This capacity is especially enhanced in tumors under hypoxia.[247] CQ can be onco-suppressive against tumor cells that activate autophagy to escape TRAIL-induced apoptosis, or escape killing by NK cells.[246,248] In the case of human cancer xenografts in mice, 75 mg/kg CQ had an immunostimulatory effect, especially once the Treg cells were depleted by the addition of cyclophosphamide. Particularly, CQ resets the tumor-associated macrophages from the immunosuppressive M2 toward a stimulatory M1 phenotype.[60] CQ also prevents the clearance of apoptotic cells by macrophages.[249] This effect could augment the immune response against tumors. In dendritic cells, autophagy that leads to MHC-I depletion decreases their efficiency in stimulating $CD8^+$ T cells against the influenza virus.[250] In an experimental system of irradiated human bone marrow cells, 40 μM CQ and a stabilized derivative of cyclophosphamide were sufficient to stimulate elimination of contaminating B-cell acute lymphoblastic leukemia cells by a selective B-cell-directed antibody coupled to an immunotoxin.[251]

However, in the presence of a fully functional immune system, low-dose CQ (10 mg/kg) could trigger potent IFN-γ-associated immune responses against irradiated tumors, thereby protecting mice from further tumor challenge.[252] Moreover, CQ has also shown the potential to prolong the survival of xenograft-bearing mice by augmenting $CD8^+$ T cells, and suppressing tumor-associated macrophages, myeloid derived suppressor cells, and Treg cells in the tumor microenvironment.[253] In parallel *in vitro* assays, CQ inhibited secretion of transforming growth factor-β, while enhancing secretion of IFN-γ. It can therefore be concluded that the balance of T-cell types in the tumor microenvironment can determine the impact of CQs on the immune response. The capacity of CQ to stimulate immune responses can be extended to eliciting antiviral as well as antitumor activity in the clinical setting.[254]

What appears consistent between the lupus and cancer studies is the critical role played not only by macrophage polarizing conditions but also by the T-helper phenotype. T cells are remarkably resistant to drugs that cause endoplasmic reticulum stress and autophagy.[255] CQ can protect against autoimmune responses by decreasing expression of both Th1 and Th2 components.[256] Suppression of Th1, albeit without the potential to inhibit Th17-driven autoimmunity, was consistent also in cultured monocyte-derived Langerhans-like cells in response to IL-1β, where 20 μM HCQ allowed them to prime CD4-negative T cells for IL-17 production, while suppressing IFN-γ production.[257] It would therefore be interesting to examine if in the immunocompetent mouse systems where CQs are immunosuppressive, this effect can be reversed by Treg cell depletion. In conclusion, even though lysosomes are an essential component of cells of the immune system, CQs can have beneficial effects, depending on the timing of administration and the cell types activated in the tumor microenvironment.

Clearly, other goals, such as the use of CQ to enhance antineoplastic drug retention, which requires high-dose and simultaneous treatment with both drugs, are not consistent with the aim to avoid immunosuppression. This is because of the time needed for the immune response to commence. The choice of administering CQ after the antineoplastic drug is the first step for mutual compatibility, and the question remains, on how long the ideal pause of treatment before CQ administration is. The 3 days needed for the restoration of IFN-γ response may be too long for cases where CQ is needed to act rapidly to enhance cell stress. One possible solution is to develop experimental treatment schemes that include both dosing with a 3-day delay, as well as dosing with simultaneous administration of two drugs. During simultaneous administration with other drugs, CQ needs to be either at a smaller dose or in a specific nanocarrier that can escape scavenging by antigen-presenting cells.

Feasibility of treating malignant tumors with CQ: strengths and weaknesses

Free CQ has limited utility as a prospective monotherapy. Individuals on long-term exposure to HCQ or CQ are not at lower risk of cancer. However, there is statistical evidence that CQs may lower the risk of metastatic cancer and death.[215] Therefore, CQ interferes with the capacity of malignant tumors to generate lethal metastases. Consistently, it was shown that CQ induces the secretion of tumor suppressor Par-4 in a p53-dependent manner.[258] Secretion of Par-4 triggered apoptosis and inhibited metastatic tumor growth.[258] This effect of CQ would be consistent with autophagy inhibition, because autophagy bestows malignant cells a metabolic flexibility that is essential to survive in niches with limited nutrient availability. Hence CQ can be expected to inhibit homeostatic adaptation of malignant cells.[37] Indeed, in triple-negative breast cancer tumors, CQ kills $CD44^+/CD24^{-/low}$ CSCs

and diminishes the tumors' ability to metastasize *in vitro* and in mouse xenografts.[259] It is important, however, to emphasize that CQs mainly affect tumor cells with stem-like features.[205] Therefore, their clinical application for cancer would be limited unless used in combination with drugs that kill cancer cells with more differentiated features.

Chloroquine cannot enter cells in an acidic microenvironment, which currently further limits its clinical application as explained in the introduction. The combination of CQ with biguanide metformin entered a clinical trial for patients with solid tumors bearing mutations in the *IDH1* and *IDH2* genes.[200] IDH-mutant gliomas might be suited for CQ treatment. These gliomas had shown a decreased capacity to generate an acidic extracellular microenvironment, an effect, however, that cannot be generalized.[260] One potential solution to decrease tissue acidity is the use of inhibitors of glycolytic flux such as dichloroacetate, which might inhibit acidosis without compromising cancer treatment.[261] Their effectiveness in pH regulation of the tumor microenvironment will still need to be empirically determined.

Given the low capacity of CQ to penetrate in cells in an acidic environment, it is imperative to develop methods that allow entry of CQ in malignant tumors *in vivo*. In addition to blocking entry of free CQ into cells, the acidic extracellular microenvironment triggers activation of the transcription factor TFEB that induces lysosomal biogenesis.[30,262] TFEB is one of the regulatory proteins that in cancer cells can become uncoupled from mechanisms that control their cytoplasmic retention. Deregulated TFEB leads to metabolic reprogramming of the cancer microenvironment.[263] *In vitro*, cancer cell autophagy and TFEB activity can be modeled in three-dimensional growth conditions.[264] In many cell types, though, especially in normal macrophage cells, TFEB is mutually antagonistic to STAT3 activity, and would thereby limit the protection of a tumor by macrophages.[265] It was shown that TFEB is activated and translocated to the nucleus via inhibition of mammalian target of rapamycin complex by CQ.[266] Activation of TFEB by CQ can induce antitumor M1 macrophage activity.[60] M1 macrophages could improve the effect of chemotherapy and lead to tumor growth regression.[267] On the other hand, in cancer cells, active STAT3 could still facilitate lysosomal cell death by inhibiting TFEB in the nucleus.[268] STAT3 is, however, also a factor critical in the M1-to-M2 activity transitions in the tumors.[269] Therefore, an intervention that differentially targets malignant cells from macrophages could have an added value.

With respect to potential drug combinations, an example of a drug that targets CSCs, and in parallel induces cytoprotective autophagy, is polyether ionophore antibiotic salinomycin. On one hand, salinomycin was shown to interfere with tumor necrosis factor and IL-6 signaling and to impair networking of transcription factors STAT1 and STAT3.[270] On the other hand, it may target the Wnt/β-catenin signaling pathway to promote differentiation and thus elimination of CSCs, while rescuing normal fibroblasts from CQ toxicity.[271] Salinomycin is a drug that must be used at strictly limited concentrations, due to its potential neurotoxicity.[272,273] This can make its synergistic antineoplastic effects with other drugs beneficial, if they decrease systemic exposure and toxicity to non-transformed cells. To this end, under the acidic conditions that inhibit CQ activity, salinomycin reaches the acidic core of multicellular tumor spheroids and impairs the autophagic flux, thereby killing malignant cells.[274] This could allow a 'first hit' against a tumor and pave the way for a combination treatment. In line with the potential of CQ for synergy with inhibitors of DNA repair, the antineoplastic effect of salinomycin is neutralized by the induction of DNA repair via DNA–protein kinase (DNA-PK) enzymes.[275] Hence, theoretically both salinomycin and CQ can be combined with DNA repair inhibitors.

DNA-PK enzymes are a group of interesting targets to inhibit, as they have the capacity to increase the ratio of IL-10 to IL-12, and thereby can inhibit Th1-driven antitumor immune responses.[276] Oncogene activation increases DNA replication stress, which can make at least a fraction of tumor cells highly dependent on DNA maintenance.[277] In addition to impairing DNA maintenance, DNA-PK inhibitors could affect immune responses at several levels, and therefore the kinetics and dynamics of adding them to a combination treatment scheme need to be studied.[276,278] The main difference between CQ and salinomycin is the capacity of salinomycin to both induce, and under some conditions inhibit the autophagic flux. Salinomycin can induce autophagy via AMPK, and in parallel inhibit autophagy at later stages: thereby salinomycin use exploits the imbalanced induction of autophagic pathway components that takes place in some cancer cell types.[275,279] Hence, the mechanisms through which salinomycin activates autophagy apparently make cancer cells prone to cell death, depending on the downstream signals it elicits. This is probably the main reason that salinomycin can sensitize some cells to CQ.

In fact, CQ can also inhibit topoisomerase II, although weakly, in addition to its lysosomotropic activity.[280] Interestingly, it was reported that CQ preferentially enhances the death of Myc-overexpressing cells, in a p53-dependent but not an ATM-dependent manner.[93] Moreover, CQ can prevent genomic instability by inhibiting etoposide (Topoisomerase II inhibitor)-induced centrosome amplification in a CDK2 inhibiting manner.[14] Similarly, CQ can be combined with other drugs causing DNA damage, such as anthracyclines, especially when using nanocarriers that enable cell penetration, leading to inhibition of the growth of human prostate cancer xenografts in mice.[45] Therefore, a combination with inhibitors

of DNA repair can be studied, as under certain conditions it might improve Th1-driven immune responses, and in parallel, provide additional cytotoxicity toward malignant cells.[143] In human breast cancer cells, a mutant adenomatous polyposis coli gene induces STAT3, an activator of DNA repair genes, which renders cells resistant to anthracyclines.[197,281] In normal tissues, STAT3 would protect from excessive Th1 activity and inflammation.[282] In normal and tumor tissue alike, the activity of STAT3 itself affects vascularity and pericyte interactions with their microenvironment, including myeloid cells.[283–286] However, unlike malignant tissue niches, normal tissue uses the intact regulatory network of STAT3 and NF-κB as a common mechanism to recover from inflammation.[147,287,288] Therefore, the activation of STAT3 by constitutive DNA damage in cancer cells could facilitate selective drug effects on the tumor nest through the circulation, while having discrete indirect effects on the local tissue microenvironment owing to the secretome of the tumor. It is therefore beneficial to dissect effects experimentally by cell-selective agents. To some extent, cellular targeting by CQs can be achieved by conjugation with specific moieties, including sugar groups, and by the generation of hybrid molecules, such as CQ-artemisinin.[20,289,290]

What is the technical maturity of the concepts of CQ use against tumors? Even though CQ is an established drug against certain conditions, its use in cancer is still in its infancy, as is evident by the clinical trials that have been publicized. The translational work of mechanistic CQ combination concepts on animal models is currently placed at an intermediate technology readiness level. Namely, as reviewed in previous sections, a number of concepts developed on the basis of induction of cell stress and under those conditions the experimental uses of CQ against tumors are gaining more attention.[43,68,102,291] Hopefully at least the most promising concepts and combination methods will ultimately reach the safety level required to enter clinical trials. On the other hand, in spite of the widely recognized dose-limiting toxicity of CQs, the technology of molecular carriers for CQs or similar agents to reprogram immune responses, with cell-selectivity and retaining direct antineoplastic activity at a low dose, is not yet close to clinical use.[292,293] In contrast to genomics, there does not seem to be a major change of research support in sight for high-capacity molecular innovation.[294–296] Furthermore, exploration of molecular concepts cannot be covered by biopharmaceutical industries, due to the fact that the concepts address essentially basic research. Research priorities, however, can change in the public sector.[297–299] Grant calls that cover the topic of drug repurposing technology to reprogram immune responses with cell-selectivity and parallel onco-suppression will be beneficial to both basic and translational research.

Conclusion

Our understanding of cancer dynamics and metabolism has improved greatly. However, there are a lot of details in the field that need to be understood to develop effective treatment options for various cancers. Based on our knowledge of the molecular mechanisms of carcinogenesis and cancer cell survival, cancer treatment concepts are also evolving. The relationship between autophagy and cancer progression has been well documented. Therefore, an approach for cancer treatment can be formulated by targeting autophagic mechanisms.

Chloroquine and its analogues are valuable tools in cancer research. They will become consolidated as antineoplastic agents as soon as our progress in cancer research helps elucidate their molecular mode of action. Research shows that under certain circumstances cancer cells develop a dependency on autophagy, either of their own contents, or on autophagy activity of stromal cells. In such cases, an organelle-selective or cell-selective and intense exposure to CQs or their derivatives could provide researchers with the opportunity to sensitize tumors to a number of antineoplastic drugs. Development of CQ-based cancer treatment options is expected to advance significantly within the next decade. Through this development, the bench-based research has the capacity to provide translational research with several interesting targets, which in the long run will certainly improve our concepts of renewing the antineoplastic armamentarium.

The best known effect of CQs is the inhibition of autophagy by increasing lysosomal pH. However, another mechanism to inhibit autophagy, via preventing autophagosome–lysosome fusion, has recently been proposed. This new mechanism should be further investigated due to its direct influence on immune response, inflammation and carcinogenesis. Concordantly, the study of uncharacterized effects of CQs may lead to an unexpected discovery of modulating factors for immunological activity, a crucial parameter for both carcinogenesis and cancer treatment.

Disclosures

All authors declare no conflict of interest.

Ethical approval

This article does not contain any studies with human participants performed by any of the authors.

Informed consent

For this type of study, formal consent is not required.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Genomic targets of chloroquine pharmacological activity. Shown is a snapshot from the query of the database DRUGSURV (http://www.bioprofiling.de/cgi-bin/GEO/DRUGSURV/start_DRUG.pl) for chloroquine.

