Chemico-Biological Interactions 386 (2023) 110750

Contents lists available at ScienceDirect

Chemico-Biological Interactions

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

Review Article

Hydroxychloroquine: Key therapeutic advances and emerging nanotechnological landscape for cancer mitigation

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ARTICLE INFO

Keywords:
Hydroxychloroquine
Nanoencapsulation
Nanoassemblies
Tumor-targeted therapy
Combined therapies

ABSTRACT

Hydroxychloroquine (HCQ) is a unique class of medications that has been widely utilized for the treatment of cancer. HCQ plays a dichotomous role by inhibiting autophagy induced by the tumor microenvironment (TME). Preclinical studies support the use of HCQ for anti-cancer therapy, especially in combination with conventional anti-cancer treatments since they sensitize tumor cells to drugs, potentiating the therapeutic activity. However, clinical evidence has suggested poor outcomes for HCQ due to various obstacles, including non-specific distribution, low aqueous solubility and low bioavailability at target sites, transport across tissue barriers, and retinal toxicity. These issues are addressable via the integration of HCQ with nanotechnology to produce HCQ-conjugated nanomedicines. This review aims to discuss the pharmacodynamic, pharmacokinetic and anti-tumor properties of HCQ. Furthermore, the antitumor performance of the nanoformulated HCQ is also reviewed

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https://doi.org/10.1016/j.cbi.2023.110750
Received 25 March 2023; Received in revised form 12 September 2023; Accepted 5 October 2023
Available online 13 October 2023

thoroughly, aiming to serve as a guide for the HCQ-based enhanced treatment of cancers. The nanoencapsulation or nanoconjugation of HCQ with nanoassemblies appears to be a promising method for reducing the toxicity and improving the antitumor efficacy of HCQ.

1. Introduction

Quinine, the alkaloid isolated from the bark of cinchona tree, was identified as the first natural agent used for malaria treatment as early as the 1600s. With 400 years of efficacy documented, quinine plays a crucial role in drug development as well with more effective synthetic quinine derivatives such as chloroquine (CQ) and hydroxychloroquine (HCQ) being developed [1]. Apart from being extensively used as an antimalarial agent, HCQ has been recognized for their effectiveness in the management of rheumatic and autoimmune diseases treatment, as well as other infectious diseases (Table 1) [2,3]. Recently, there has been a bloom of interest in repurposing HCQ for the prophylactic treatment of SARS-coronavirus 2 (SARS-CoV-2) infection owing to its involvement in treatment of inflammatory rheumatic diseases (Table 1) [3,4].

Notably, HCQ also represents one of the potential repurposed drugs for cancer treatment, either in monotherapy or combination therapy (Table 1). Many preclinical and clinical studies have reported that HCQ could exhibit anti-cancer effect via autophagy inhibition [44,45]. In normal cells, autophagy is a catabolic process which permits cells to recycle intracellular organelles and macromolecules. However, in some malignancies, autophagy could act as the tumor promoter and survival mechanism [46]. Even though HCQ and CQ are the only clinically approved autophagy inhibitor, the exact mechanism of autophagy inhibition is still poorly understood. As a weak base, it is proposed that both CQ and HCQ regulate lysosomal acidification via accumulation in the lysosome, thereby inhibiting the maturation of lysosomes and autophagosomes [47]. As compared to CQ, HCQ was preferred to be used as an autophagy inhibitor in clinical trials due to lower toxic effects [48].

Even though pre-clinical and clinical data have proven the efficacy of HCQ in cancer treatment, the clinical translation is still limited due to its low bioavailability, non-specific targeting and adverse side effects such as gastrointestinal and retinal toxicities [18,49]. In order to overcome the shortcomings, several approaches such as molecular modification and development of nanoformulation has been proposed. The latter approach has been extensively studied as nanoparticles could enhance the efficacy via targeting, controlled release, and minimized toxicity. This review focuses on the current applications of HCQ in preclinical studies and clinical practices, with specific emphasis on the effect of autophagy inhibition in cancer as well as the use of nanoparticle-based therapeutic approaches to improve the pharmacokinetic and pharmacodynamic properties of HCQ. The intrinsic challenges and future strategies for the employment of HCQ will be discussed.

2. Hydroxychloroquine (HCQ): pharmacokinetics and pharmacodynamics

2.1. Origin and structure

Quinine ($C_{20}H_{24}N_2O_2$) was the first alkaloid extracted and identified from the cinchona tree bark in a large-scale screening program for antimalarial research led by the U.S. OSRD (Office of Scientific Research and Development) during World War II [3]. Since then, more quinine-derived synthetic compounds such as chloroquine (CQ) and hydroxychloroquine (HCQ) have been developed [3]. HCQ is claimed to be more water soluble and less lipophilic than CQ due to the presence of a hydroxyl group at the side chain tertiary N (Fig. 1). HCQ has the molecular formula of $C_{18}H_{26}ClN_3O$ and molecular weight of 335.9 g/mol, and exists in a pair of enantiomers, designated as R(−) and S(+) isomers [50,51].

2.2. Bioavailability: Absorption, distribution, metabolism, and elimination

Fig. 2A summarizes the absorption, distribution, metabolism, and elimination of HCQ in the human body. HCQ is generally administered orally as a sulfate salt (hydroxychloroquine sulfate; HCQS) and is rapidly absorbed in the upper gastrointestinal tract with bioavailability of 0.7–0.8 [52]. Oral absorption is claimed to be unaffected by food consumption. Like other 4-aminoquinoline compounds, HCQ is a weak base that tends to accumulate in acidic compartments such as lysosomal vesicles [53]. Interestingly, it is important to note that HCQ does not accumulate in fatty tissues [54]. As HCQ could be repurposed to be used in anti-cancer therapy, bioaccumulation of HCQ in the tumor site might be affected as some tumors are surrounded by fat cells. To overcome this, enhanced drug delivery such as nanocarriers could be suggested for HCQ in cancer chemotherapy.

2.3. Modes of action

As HCQ has high affinity for intracellular lysosomal and endosomal space due to its basic properties, it exhibits immunomodulatory effects to treat autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus. [53–55] Several possible modes of action of HCQ have been elucidated (Fig. 3), including the inhibition of autophagy and modulation of Toll-like receptor (TLR) signaling pathway. Autophagy inhibition by HCQ occurs through the elevation of lysosomal pH, thus inhibiting the maturation of lysosomes and autophagosomes utilized by antigen-presenting cells (APCs) to display short peptides on major histocompatibility complex (MHC) class II for detection by $CD4^+$ T cells [38,56,57]. Consequently, $CD4^+$ T lymphocytes activation is inhibited and the production of pro-inflammatory cytokines is suppressed [38,58,59]. On the other hand, HCQ modulates endosomal TLR signaling via antagonization of the TLRs (i.e. TLR3, TLR7, TLR8, and TLR9), thereby suppressing immune activation [60].

Another potential mode of action of HCQ is the modulation of cyclic GMP-AMP (cGAMP) synthase (cGAS)/stimulator of Type 1 inteferon (IFN) genes (STRING) signaling pathways [61]. Upon binding to cytosolic DNA, enzyme cGAS is activated, leading to the formation of small second messenger cGAMP and initiation of STRING transcription through the transcription factor IFN regulatory factor 3 (IRF3) [62,63]. As HCQ blocks double stranded DNA stimulator of the cGAS-STING pathway, subsequent pro-inflammatory signaling activation and production of cytokines are attenuated.

Taken together, these evidences suggest that HCQ downregulates the production of pro-inflammatory cytokines by interrupting TLR-mediated immune responses which are likely the central mechanisms of action, as well as cGAS-STING pathway (Fig. 4). Such mechanisms support the hypothesis that HCQ is likely to suppress tumor progression from over-activation of the immune system triggered by tumoral cells.

2.4. Drug-drug interactions, adverse effects, and toxicity

Previous studies have confirmed that co-medication of HCQ with tamoxifen, the cytotoxic agent for breast cancer therapy, causes irreversible toxic retinopathy [64,65]. As the administration of tamoxifen alone would cause minor retinal toxicity, the co-administration of HCQ would further complicate the toxicity due to cumulative effect. Studies have also found out that proton-pump inhibitors, such as omeprazole, lansoprazole, pantoprazole, esomeprazole and rabeprazole are interfering with the oral absorption and oral bioavailability of HCQ [66].

Table 1
Past and current indications and applications of HCQ.

Category	Effect	Disease/disorder	Possible mechanism	Outcome/finding of study	References
Infectious diseases	Antimalarial	Malaria caused by *Plasmodium vivax*	Concentrating in the acid vesicles of the parasite and by inhibiting polymerization of heme.	Prophylactic agent against malaria.	[5]
	Antibacterial	Chronic Q fever endocarditis	Increase cell pH in phagolysosomes to stop replication.	Shortening of the duration of therapy and reduction in the number of relapses.	[6]
		Q fever (*Coxiella burnetii* infection)	Increase pH above 5.5 in phagolysosomes to stop replication.	Most effective treatment in combination with doxycycline.	[7]
		Whipple's Disease (*Tropheryma whippeli* infection)	Alkalization of the vacuoles induced by hydroxychloroquine.	Combination with doxycycline for preventing relapses	[8]
	Antiviral	Human immunodeficiency virus (HIV)-1	Inhibition of post-translational modification of glycoprotein 120 (gp120) in T cells and monocytes.	Reduction in HIV-1 RNA load in plasma.	[9–11]
		Human immunodeficiency virus (HIV)-1	Inhibition of post-translational modification of glycoprotein 120 (gp120) in T cells and monocytes.	Decreased HIV-1 replication and increased $CD4^+$ numbers.	[12]
		Human corona virus (SARS-CoV-2)	Suppress the increase of immune factors (cytokines IL-6 and IL-10).	$EC_{50} = 0.72$ μM *in vitro*.	[13]
		Human corona virus (SARS-CoV-2)	Inhibit endosomes maturation and block entry of virion.	IC_{50} 249.50 μM in Vero E6 cells with increased number and size of endolysosomes.	[14]
		Human corona virus (SARS-CoV-2)	–	Synergistic with azithromycin caused relative viral inhibition of 97.5 % with cytopathic effect at 60 h post infection.	[15]
		Zika virus	Inhibition of NS2B-NS3 protease	Inhibition constant (K_i) of 92.34 ± 11.91 μM.	[16]
		Dengue virus	Induce ROS and mitochondrial antiviral signaling protein (MAVS).	Expression of IFN-related antiviral proteins and certain inflammatory cytokines.	[17]
Non-Infectious disease	Immune-related	Rheumatic arthritis	Diminish the formation of peptide-MHC protein complexes required to stimulate $CD4^+$ T cells and result in down-regulation of the immune response against autoantigenic peptides.	Combination with methotrexate effectively reduced the occurrence of acute hepatic effects and nodulosis.	[18]
		Rheumatic arthritis	Diminish the formation of peptide-MHC protein complexes required to stimulate $CD4^+$ T cells and result in down-regulation of the immune response against autoantigenic peptides.	Reduced swollen joint count, pain and ACR20 response.	[19]
		Rheumatic arthritis	Diminish the formation of peptide-MHC protein complexes required to stimulate $CD4^+$ T cells and result in down-regulation of the immune response against autoantigenic peptides.	Good therapeutic response with reduced both clinical and laboratory signs of activity.	[20]
		Rheumatic arthritis	Inhibits proinflammatory cytokines.	Significant reduction in the IL-6, IL-17 and IL-22	[21]
		Systemic lupus erythematosus	–	Protect against low bone mineral density in corticosteroid treated patients with SLE.	[22]
		Systemic lupus erythematosus	–	Decrease abnormal levels of cytokines. Interleukin-6 (IL-6), soluble CD8 and soluble IL-2 receptors (sIL-2R).	[23]
		Systemic lupus erythematosus	Inhibits proinflammatory cytokines.	Significant reduction in the IL-6, IL-17 and IL-22.	[21]
	Neurological	Multiple sclerosis	B-cell proliferation was mitigated, reduced TNF-α production of activated microglia in culture.	Reducing experimental autoimmune encephalomyelitis (EAE) severity in acute and chronic EAE	[24]
	Antineoplastic	Acute myeloid leukemia	Induction of p53-dependent cell death.	Inhibition of malignant cell growth, viability.	[25]
		B-chronic lymphocytic leukemia	Activation of caspase-3.	Inhibition of malignant cell growth, viability.	[26]
		Breast cancer	Increase protein acetylation.	Inhibition of malignant cell growth, viability.	[27]
		Antiphospholipid syndrome (APS)	Inhibition of platelets activation by aPL antibodies, reducing binding of aPL-beta2-GPI to antiphospholipids and disruption of annexin A5 by aPL antibodies.	Reduces aPL-induced thrombosis.	[28]
		Antiphospholipid syndrome (APS)	Restored the binding of AnxA5 to phospholipids to the levels of control IgGs.	Protect the AnxA5 anticoagulant shield from disruption by aPL antibodies on phospholipid bilayers.	[29]
Metabolic	Anti-hyperglycemic	Diabetes mellitus	Decreasing insulin clearance and increasing secretion of C-peptide.	Daily dose of insulin reduced by 30 % in the subgroup treated with hydroxychloroquine	[30]
		Diabetes mellitus	Greater stimulated C-peptide response	Decrease of 1.02 % in the hemoglobin A1c compared with placebo.	[31]
	Anti-lipidemia	SLE-associated	Reduce the proteolysis of low-density lipoprotein (LDL) receptors.	Lower serum cholesterol and low-density lipoprotein levels.	[23]

(*continued on next page*)

Hence, the interactions between HCQ with other drugs should be carefully monitored in patients receiving concomitant treatment. Although HCQ is claimed to be generally well tolerated, there is still a number of adverse effects that should be taken into consideration by healthcare providers, as listed in Table 2. Nevertheless, HCQ has a better safety profile in modern medicine for most non-malarial and non-rheumatic indications.

3. antitumor properties of HCQ

3.1. Hallmarks of tumor progression

In 2018, cancer has claimed the lives of estimated 9.6 million people with 18.1 million new cases diagnosed worldwide, reflecting about 1 in 6 deaths due to cancer. It has become the world's second leading cause of death after cardiovascular diseases [74]. Lung and breast cancers remain the top most prevalent cancer in both developed and developing countries, accounting for 11.6 % of the total cancer incidence burden [74,75]. Colorectal, stomach, liver and lastly, prostate cancer are the next most frequently diagnosed cancers globally [75]. Other than genetic defects, other lifestyle factors such as smoking, alcohol use, physical inactivity, unhealthy diet, as well as chronic infections such as bacterial and viral infections have been contributed to the increase of cancer incidence [74,76].

Tumors have been long regarded as highly heterogeneous and complex tissues in which mutant cancer cells recruited the residents and normal cell types to serve as contributors toward a neoplastic phenotype (abnormal and excessive growth) [77]. The hallmarks of cancer initiation include a series of biological events involving the evasion of immune system, tumor-induced inflammation, resistant of cell death and growth suppressors, uncontrolled cell proliferation, replicative immortality, activation of invasion and metastasis, genome instability and mutation, and angiogenesis [78,79]. From here, we could postulate that seven out of the eight hallmarks has demonstrated the involvement of stromal cells from the TME. By understanding the tumorigenesis pathways, we could rule out some possible causes of resistance and facilitate the development of more effective therapeutic approaches [77].

3.2. TME induced autophagy

Autophagy, the lysosomal degradation pathway, has claimed to be the key player in modulating tumorigenesis, tumor–stroma interactions and cancer therapies [80]. The term autophagy derived from Greek meaning "eating (phagy) of part of the cell itself (auto)" [47]. Relationship between autophagy and cancer is particularly complex as autophagy modulates the interaction of tumor cells with components of its microenvironment (TME) such as non-cancer cells (e.g., immune cells, stromal cells, endothelial cells, and adipocytes) and various mediators (e.g., cytokines, chemokines, growth factors, and humoral factors) that work together to support cancer growth [46].

Autophagy may have dual functions in cancer (Fig. 5), paradoxically cytoprotective or cytotoxic/cytostatic in the promotion and prevention of tumor progression, respectively, depending on the environment and the different stages of tumorigenesis [44,81]. Under normal circumstances, autophagy in normal cells is a potent anti-inflammatory or tumor suppressor mechanism via the degradation of pro-inflammatory regulators such as the inflammasome complex [82,83]. It protects cells that suffer in an unfavorable microenvironment by elimination of damaged proteins or organelles. Nevertheless, autophagy could lead to apoptosis (type II programmed cell death) if cellular damage is irreversible [84]. Whereas in TME, autophagy plays a dual role in regulation of inflammation response. As tumor suppressor mechanism, autophagy regulates the inflammatory TME through the production and release of critical inflammatory cytokines, such as high-mobility group box 1 (HMGB1), interleukin (IL)-6 and recruitment of inflammatory cell [85]. Besides, autophagy has been demonstrated to regulate antigen presentation to and by immune cells, which may then contribute to tumor--localized immune responses [86]. Autophagy directly mounts a T-cell memory response by regulates T-cell and natural killer cell activity [86]. Conversely, as tumor promoting mechanism, autophagy contributes by supplying substrates and nutrients as energy to cancerous cells under inadequate oxygen supply or any metabolic stress within the TME [87].

3.3. Preclinical evidence of HCQ in cancer

As autophagy could dysregulate cellular functions, pharmacological approaches to up-regulate or down-regulate this pathway has been

Table 1 (*continued*)

Category	Effect	Disease/disorder	Possible mechanism	Outcome/finding of study	References
		SLE-associated	Removal of cholesterol-rich microemulsions by LDL receptor from plasma.	Lower total and LDL cholesterol.	[32]
Cardiovascular		Cardiac ischemia/ reperfusion	Enhancement of ERK1/2 phosphorylation.	Decrease in infarct size observed in an *in vivo* model of myocardial I/R injury in hydroxychloroquine treated rats.	[33]
		Cardiovascular disease (RA-associated)	–	72 % reduction in the risk of incident cardiovascular diseases.	[34]
Miscellaneous	Skin disease	Cutaneous sarcoidal granulomas	Suppression of the inflammation.	Transient improvement in dyspigmentation.	[35]
		Kikuchi-Fujimoto disease	–	–	[36,37]
		Sjögren's syndrome	–	Decreased rheumatoid factor production, decreased IgG *anti*-Sjögren's syndrome-associated antigen B autoantibody.	[38]
		Eosinophilic fasciitis	–	Improvement of symptoms and complete resolutions.	[39]
		Dermatomyositis	–	Adjuvant therapy of patients with cutaneous lesions of dermatomyositis.	[40]
		Granuloma annulare	Delayed type Th1 hypersensitivity reaction.	Improvement of the cutaneous manifestations after 15 days, complete remission within 6–7 week with no relapsed.	[41]
		Oral lichen planus	Anti-inflammatory effects of stabilizing lysosomal membrane, inhibition of prostaglandin synthesis and other hydrolytic enzymes.	Pain relief and reduced erythema.	[42]
		Lupus panniculitis	–	Flattened nodules without leaving a depressed scar and without recurrence.	[43]

investigated for various diseases including cancer. Inhibition of autophagy has gained attention as it may be a good strategy for treating some cancers [88]. Studies has proven that autophagy could act as a protective and resistance mechanism against chemotherapy treatment as well as inducing autophagy-mediated cell death mechanism. Even so, the role of autophagy as a target for cancer still remains controversial. First, most of the studies demonstrated that the cytotoxic effect was not solely due to autophagy inhibition, but also autophagy independent cell death and another lysosomal-dependent effect [88]. Second, there is still a central question of whether autophagy could represent a true or direct cell death mechanism during chemotherapy [88].

However, the anti-neoplastic effects induced by HCQ are not directly due to autophagy but through modulation of autophagy pathway [88, 89]. As a lysosomotropic agent, HCQ indirectly inhibited autophagy via reduction of lysosomal acidity, followed by blocking the fusion between autophagosomes and lysosomes [90]. In fact, CQ and its derivatives are the only modulators of autophagy approved by the US Food and Drug Administration (FDA) [91]. Among the CQ derivative, HCQ is the preferred analog due to its enhanced potency and minimized side effects. Therefore, clinical studies focusing on the HCQ as a promising autophagy inhibitor for cancer treatment are constantly increasing. The combination use of HCQ in conventional chemotherapy is being investigated in clinical trials as it is able to sensitize tumor cells and augment the efficacy of the existing therapies [92].

Based on the clinical trials, the HCQ could act as a promising chemosensitizer and immune regulator for cancer chemotherapy with some not significant recurring toxicities such as fatigue, anorexia and nausea reported in both monotherapies and combined therapies [93–97]. Even though autophagy inhibition by HCQ could be a novel strategy approach, it is still unclear of the effective dose of HCQ to inhibit autophagy completely in human tumour. The section below summarizes the anti-cancer efficacy of HCQ, either as monotherapy or combination therapies, based on the pre-clinical and clinical evidences.

3.3.1. *HCQ monotherapy: case reports*

Lyu et al. (2020) showed that intraperitoneal injection of 80 mg/kg daily of HCQ for 30 days has significantly decreased the PC-3 tumor (lung adenocarcinoma) of xenograft nude mice via modulation of STAT3/FoxO3a/$p27^{Kip1}$ signaling pathway [48]. Another study carried out by Rosenfeldt and colleagues showed that the monotherapy of HCQ (60 mg/kg daily) by intraperitoneal injection has inhibited the autophagy and blocked the tumor progression of pancreatic ductal adenocarcinoma in in $Kras^{G12D/+}$ $p53^{+/+}$ mice [98].

3.3.2. *HCQ as adjuvant agent in cancer therapy (combined therapy): case reports*

Apart from monotherapy, the efficacy of HCQ has also been investigated when combined with other cancer treatment approaches such as chemotherapeutic agents, small molecule kinase inhibitors, monoclonal antibodies, hormone therapies and radiation therapy. An *in vivo* xenograft mouse model of melanoma treated with HCQ and mammalian target of rapamycin (mTOR) inhibitor temsirolimus (CCI-779) was investigated by Xie and colleagues [99]. Data demonstrated the synergistic effect of HCQ and CCI-779 is exhibited in human melanoma UACC903 tumor xenograft nude mice via inhibition of late-stage autophagy, accumulation of autophagosomes and induction of apoptosis. The CCI-779-induced autophagy contributes to the resistance of melanoma cells, meanwhile the addition of HCQ reverses drug-resistance and sensitize tumor cells to anticancer treatments [99]. In another study, sensitization of breast cancer (JIMT-1) cells to gefitinib in the presence of HCQ has also been reported. Both *in vitro* and *in vivo* data are consistently showing that combination treatment with gefitinib and HCQ has significantly increased ($p < 0.05$) cell death, mainly due to the inhibition of autophagy [100]. Results suggested that adequate concentration of HCQ shall present in tumor tissue in order to modulate the autophagic response during peak exposure to the autophagy-promoting drug [100]. Further work has also been done to investigate the correlation between lysosome inhibition and the sensitization effect caused by HCQ in non-small cell lung carcinoma (NSCLC). In this effort, the lungs of the mice were pre-instilled with HCQ, followed by a tail vein injection of doxorubicin (DOX) [101]. Notably, HCQ significantly enhances the chemotherapeutic efficacy for NSCLC both *in vitro* and *in vivo* through a striking inhibition of tumor growth. HCQ elevates the lysosomal pH (P-glycoprotein in lysosomes induces the sensitization) and fosters the transition of M2 tumor-associated macrophages (TAMs) into M1-like macrophages, leading to $CD8^+$ T cell infiltration into the TME and anti-tumor immune response [101]. These findings suggest that HCQ could act as a promising chemo-sensitizer and immune regulator for cancer chemotherapy.

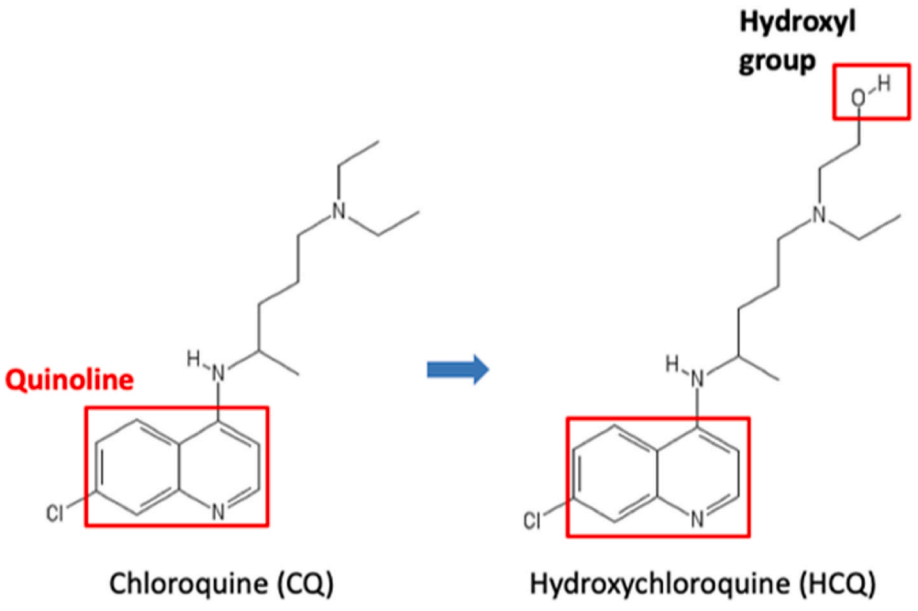

Fig. 1. Chemical structure of CQ and HCQ.

3.4. Human data of clinical trials on HCQ

To date, around 40 early phases clinical trials with HCQ as a component of the anti-cancer treatment regimen have been initiated and completed. In these clinical trials, HCQ are given either alone or in combination with conventional chemo-, radio- or immunotherapeutic regimens to increase the efficacy, increase the sensitization and to limit drug resistance [47]. The past and current phase I and phase II clinical trials involving HCQ for cancer treatment are detailed in Table 3. Even though the efficacy of the monotherapy HCQ could be observed in both cell culture and animal cancer models, majority of the clinical trials focuses on the combination effects due to the chemo-sensitizing properties of HCQ. Moreover, the combination therapies of HCQ showed promising results in both solid and hematological malignancies. Even so, it is suggested that the combination therapy should be used and monitored closely as the HCQ could inhibit the autophagy in normal cells, leading to possible unwanted adverse effects. The section below discussed further on some examples of clinical trials of HCQ.

3.4.1. HCQ alone in refractory pancreatic cancer

As a standalone therapeutic intervention, HCQ failed to demonstrate beneficial effects in a cohort of 20 patients with metastatic pancreatic cancer in a phase II study [102]. This trial recruited 10 patients who received HCQ twice daily with 400 mg and another 10 patients who received 600 mg daily. It was worth to note that only 2 did not experience progressive disease at 2 months, one patient experienced grade 3 and 4 lymphopenia, and one had grade 3 and 4 transaminitis. Several mechanisms may explain the lack of efficacy and consistency for HCQ. It is postulated that the autophagy inhibition alone may not be sufficient to reduce the tumor growth or/and higher doses may be required to inhibit autophagy within the deadliest metastatic human pancreatic cancer.

3.4.2. erlotinib and HCQ in epidermal growth factor receptor (EGFR) inhibitor-treated NSCLC

A phase I dose-escalation trial of either HCQ alone or combination with erlotinib was conducted in patients with EGFR-mutant non-small cell lung cancer who had previously treated with EGFR-inhibitor therapy [103]. Twenty-seven patients were treated, eight with HCQ alone and 19 with HCQ plus erlotinib. One patient had a partial response and 4 out of 19 patients had stable disease. No dose-limiting toxicity or adverse effect related to administration of HCQ was reported. HCQ has exhibited efficacy by decreasing intralysosomal degradation of phosphorylated EGFR, thereby activating the apoptotic pathways and potentiating the antitumor activity of erlotinib.

3.4.3. HCQ in conjunction with radiation therapy and temozolomide in glioblastoma multiforme

In a phase I/II trial conducted in glioblastoma multiforme patients, HCQ was proven to augment the efficacy of DNA-damaging radiation

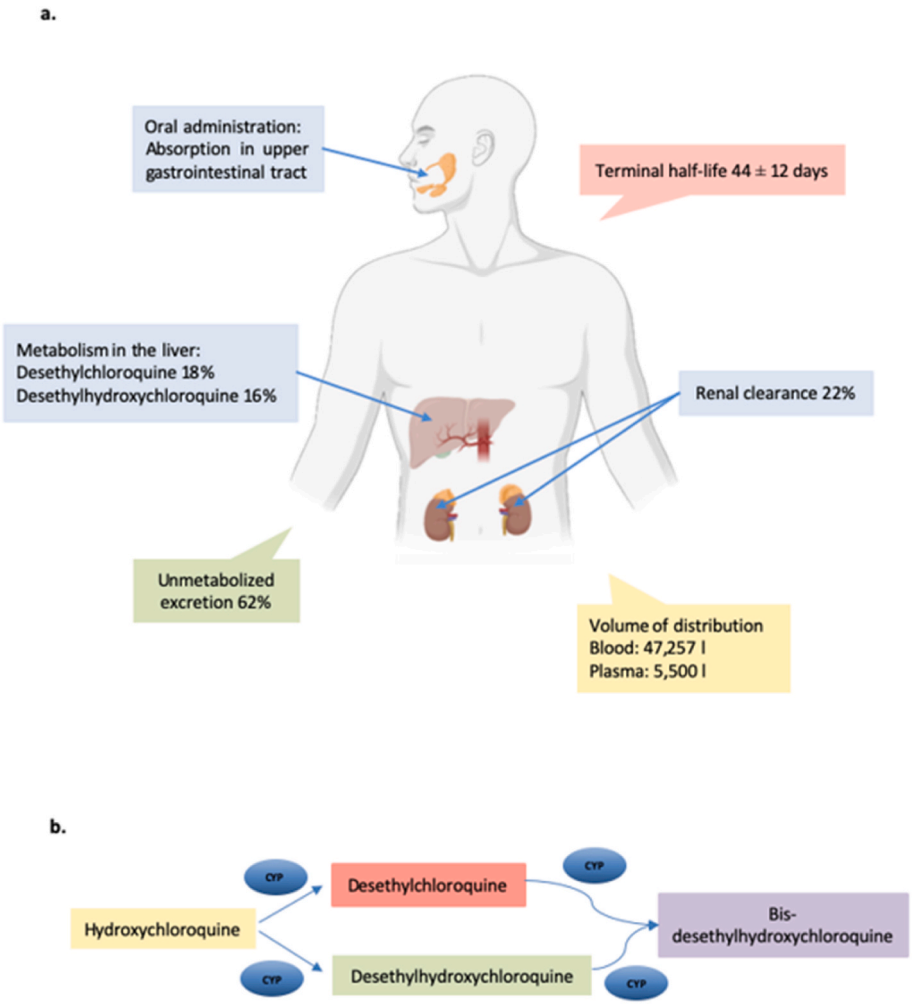

Fig. 2. Pharmacokinetics of HCQ. (a) Overview of the absorption, distribution, metabolism and elimination of HCQ in the human body. HCQ has a large volume of distribution in blood and long half-life. (b) Cytochrome P450 (CYP) enzymes catalyze dealkylation of HCQ. Desethylchloroquine is an immediate downstream product of CYP-mediated dealkylation, along with desethylhydroxychloroquine and bisdesethylchloroquine.

therapy and temozolomide (TMZ). As a autophagy inhibitor, HCQ has significantly increased the autophagic vacuoles (AV) and LC3-II in patients' peripheral blood mononuclear cell (PBMC) at maximal tolerated dose of 600 mg daily [95]. However, at dose higher than 600 mg per day, the autophagy inhibition was not consistently achieved with no survival prolongation found.

3.4.4. HCQ with dose-intense temozolomide in melanoma

Similarly, this phase 1 trial has proven that HCQ was able to augment the cell death caused by the alkylating agent TMZ in patients with advanced solid tumors. Forty patients with majority of them suffered from metastatic melanoma (73 %) received 200–1200 mg of HCQ and dose-intense TMZ daily for one to two weeks [94]. HCQ has proven to significantly increase the autophagic vacuoles (AV) in patients' PBMC. With some not significant recurring toxicities such as fatigue, anorexia and nausea reported, the study suggested that the recommended dose for phase II study would be 1200 mg daily to further study on the efficacy of this combination treatment.

3.4.5. HCQ and temsirolimus in advanced solid tumors and melanoma

This dose-escalation study has included 27 patients with advanced solid malignancies followed by a cohort expansion at the top dose level in 12 patients with metastatic melanoma [93]. High dose of HCQ (1200 mg per day), in combination with the mTOR inhibitor temsirolimus (TEM), induced autophagy inhibition and increased autophagic vacuole in PBMC and tumor biopsies in patients. Overall, the combination of HCQ and TEM was well tolerated with 14 patients achieved stable disease with common grade 1 and 2 toxicities included anorexia, fatigue, and nausea. It was demonstrated that HCQ can modulate autophagy in surrogate tissues and tumor tissues.

3.4.6. HCQ with histone deacetylase (HDAC) inhibitor vorinostat (VOR) in advanced solid tumors

Another phase 1 dose-escalation clinical trial has demonstrated the HCQ was able to augment the efficacy of histone deacetylase inhibitor known as vorinostat (VOR) [104]. The study involved 27 patients with advanced solid tumors who received HCQ (600 mg) in combination with VOR (400 mg) daily for 22 days. Study claimed that the combination treatment was efficacious and well tolerated with some common side effects observed such as fatigue and gastrointestinal toxicities. Even though autophagy inhibition by HCQ could be a novel strategy approach, it is unclear that at what dose will the HCQ inhibit autophagy completely in human tumour.

3.4.7. HCQ and bortezomib in relapsed/refractory myeloma

Vogl et al. (2014) detected an increase in vacuole formation in bone marrow plasma cells but not PBMC in a phase I trial involving 25 patients with relapsed and refractory multiple myeloma [105]. HCQ was given at 600 mg twice daily in addition to standard doses of proteasome inhibitor bortezomib (BOR). Ten patients had a period of stable disease with dose-related gastrointestinal toxicity and cytopenias. As compared to protein degradation alone, the study suggested that the addition of autophagy inhibitor could further improve the treatment efficacy in solid tumors.

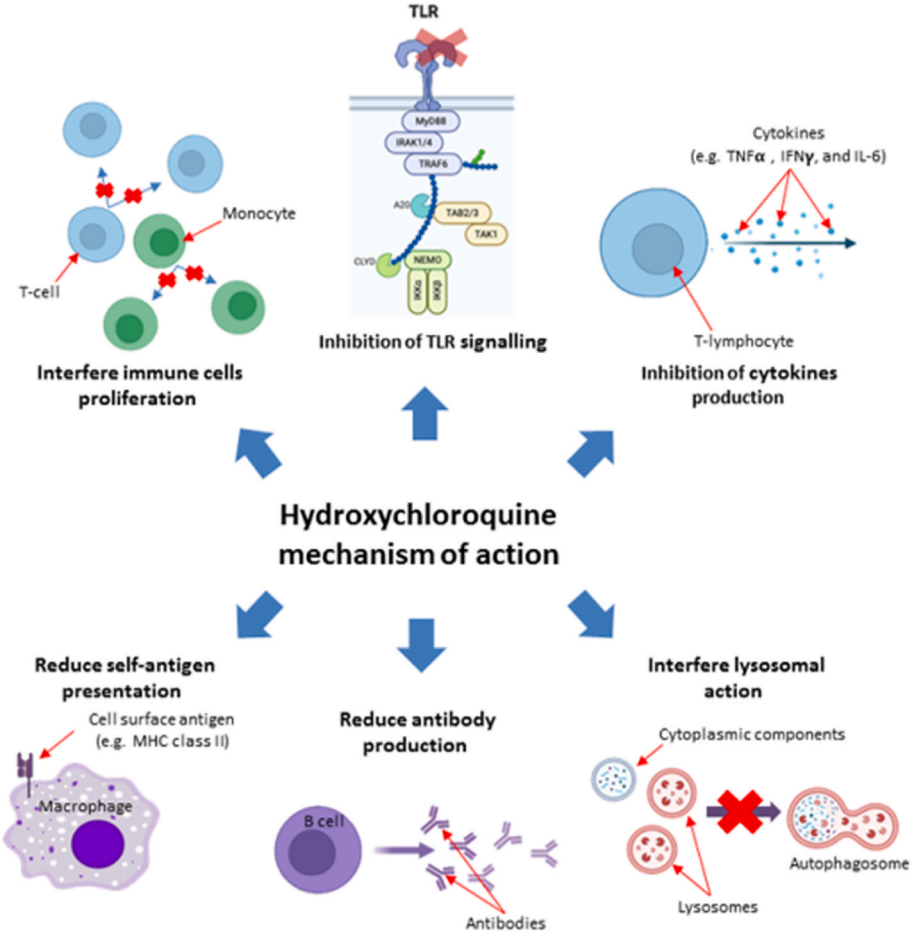

Fig. 3. The immunomodulatory effects of HCQ via decrease in immune activation, inhibition of autophagolysosome, inhibition of monocytes and T cells proliferation, modulation of Toll-like receptor (TLR) signaling, inhibition of antigen presentation and blocking of lysosomal acidification.

3.5. Challenges and current limitations of HCQ in cancer therapies

Based on preclinical and clinical studies, HCQ was proven to be promising autophagy inhibitors that could be potentially used in the cancer chemotherapy. However, the clinical translation of HCQ is limited by its bioavailability and non-specific distribution. Moreover, the therapeutic effect of HCQ is also affected by its inability to cross tumor cell membranes effectively in the acidic TME [106]. Therefore, encapsulating HCQ into drug delivery carrier such as nanoparticles to ensure maximal drug exposure to tumor cells and minimal drug circulation through the body might be a good approach to overcome the aforementioned limitations. On top of enhancing efficacy, the toxicity of

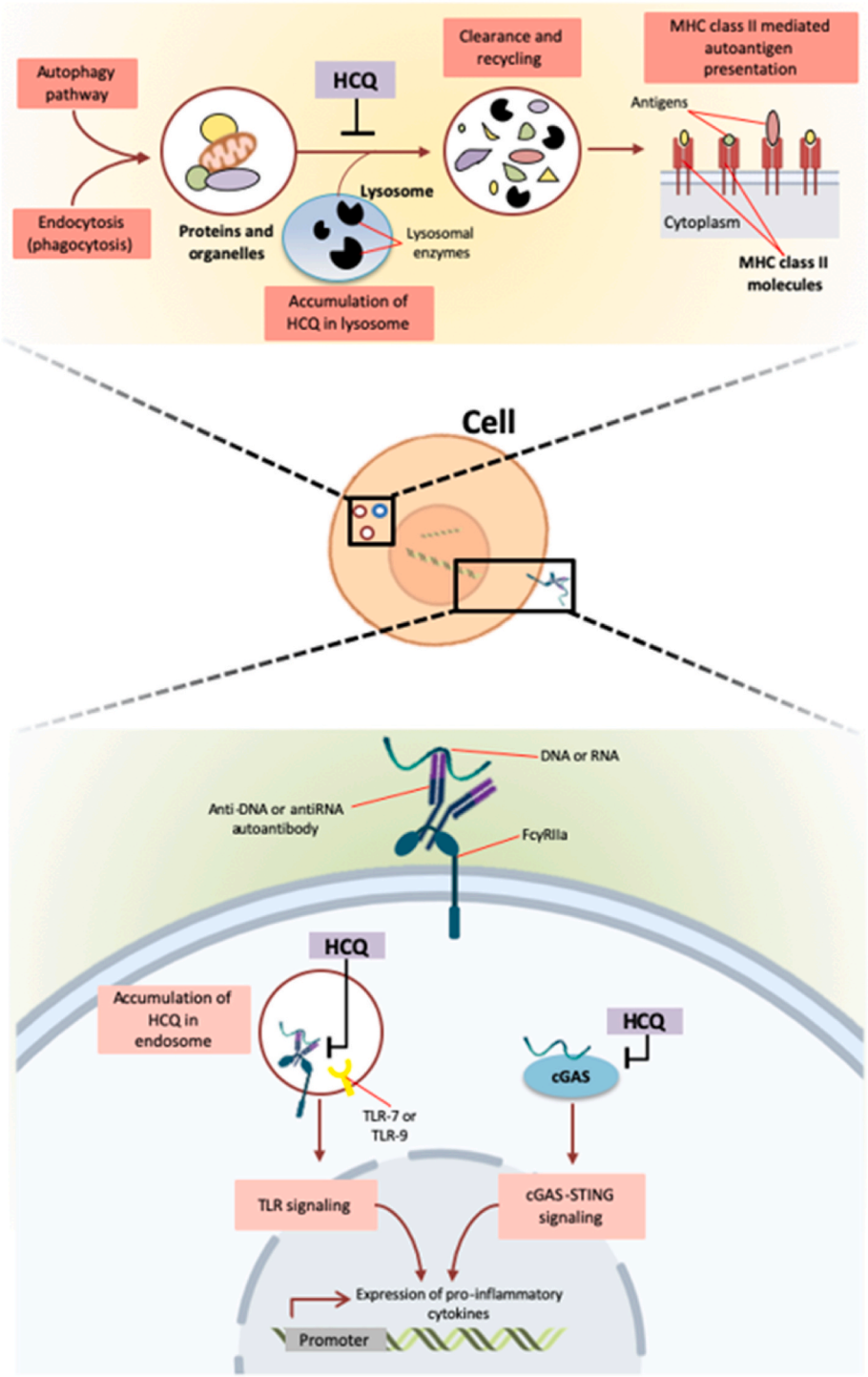

Fig. 4. Potential molecular immunomodulatory mechanisms of HCQ. (top) HCQ enters cell along a pH gradient and accumulates inside the lysosomes. In lysosomes, it increases the lysosomal pH and blocks degradation of vesicle contents via endocytosis, phagocytosis or autophagy pathway. Inhibition of lysosomal activity also downregulates MHC class II-mediated autoantigen presentation to T cell. (bottom) HCQ can also accumulate in endosomes. This drug can alter the endosomal pH and interrupt TLR-ligand binding, thus inhibiting TLR signaling. It also inhibits the activity of nucleic acid sensor cyclic GMP-AMP (cGAMP) synthase (cGAS) binding to cytosolic DNA and downregulates cGas-stimulator of interferon gene (STING) signaling. Eventually, HCQ can reduce the production of pro-inflammatory cytokines, including type I interferons.

Table 2
Known adverse effects of HCQ.

Biological system	Adverse effects	References
Gastrointestinal	Nausea, vomiting, diarrhoea, abdominal discomfort	[67,68]
Cardiovascular	Ventricular tachycardia, conduction disturbances, congestive heart failure, cardiomyopathy, leukopenia, thrombocytopenia	[4,69,70, 67]
Musculoskeletal	Myopathy associated with mild peripheral neuropathy	[71]
Cutaneous	Rashes, itching, changes in pigmentation, eczematous eruptions, photosensitivity, alopecia	[67,69]
Ocular	Bull's eye maculopathy	[72,73]
Auditory	Ototoxicity	[67]

the drug could be minimized through targeted drug delivery.

4. | Nanoencapsulation of HCQ in nanoassemblies for tumor-targeted therapies

Recent advances in nanotechnology-mediated drugs delivery systems offers new hopes to fight cancer with innovative, personalized, selective and more efficient therapeutic agents by overcoming biological barriers or shortcomings usually encountered in traditional cancer therapies [107]. Traditional cancer therapies primarily includes surgery, chemotherapy and radiotherapy. In many cases, surgery can hardly remove cancer cells completely and damage can occur to nearby healthy tissue around a tumor during surgery [108]. While many other cases, both chemotherapy and radiotherapy may have severe side effects on normal cells [108]. Besides, cancer cells can have both acquired and intrinsic resistances to chemotherapy treatment due to the dynamic changes in the hostile TME [109]. In view of this, more specific and efficient therapeutic strategies are needed to achieve satisfactory treatment outcomes. One of the biggest challenges in the formulation of nanomedicine is developing a drug delivery system to transfer drugs, proteins, enzymes or antibody into specific target sites without affecting healthy tissues [110]. Nowadays, nanomedicine and nano-delivery systems are rapidly developing science to serve as means of diagnostic tools or to deliver therapeutic agents.

The lysosomotropic agent HCQ can inhibit autophagy to some extent and has already been used in several preclinical and clinical trials as a type of chemotherapy sensitizing agent [111]. Although these findings highlight the fact that targeting autophagy is of importance in the treatment of cancer, there are several drawbacks associated with HCQ. These including *in vivo* instability, poor bioavailability, issues with target-specific delivery, and tonic effectiveness, and probable adverse effects of drugs. Pharmacokinetic analyses with HCQ also demonstrated large inter- and intrapatient variability. Nanoassemblies/nanocarriers have demonstrated great potential in delivery of autophagy modulators owing to their physical, chemical, and biological aspects such as nanoscale sizes, high surface area to volume ratio, favourable drug release profiles and targeting modifications (Fig. 6) [112]. Encapsulating HCQ in nanoassemblies could therefore mean a major step forward of HCQ in all pathological conditions, especially cancer as nanoassemblies have often proven successful at targeting drugs to tumors. To date, many efforts have been devoted to the nanoencapsulation of HCQ for antitumor therapies, which displayed the sound improvement in delivery efficiency to tumor site [111,113]. Some of the previously reported nanoassemblies/nanocarriers for HCQ include liposomes, inorganic nanoparticles, small molecular assemblies and polymeric assemblies (see Table 4). The nanoencapsulation strategies that allows the reduction of the dosage content of HCQ upon its employment for antitumor enhancement, will be discussed thoroughly in this section.

4.1. Liposome

Liposome is sphere-shaped vesicle formed through the self-assembly of amphiphilic phospholipids. They are generally comprising one or more lipid bilayers surrounding aqueous units where the hydrophilic head groups are oriented along the internal and external aqueous phases [127]. Such a unique structure enables them to carry bioactive of both hydrophilic and hydrophobic nature at the same time for drug delivery. Moreover, liposomes can be functionalized via the incorporation of the various stimuli-responsive moiety to provide them the tumor-targeting properties [128]. These features thus promote the employment of liposomes for controlled drug delivery applications. In fact, liposomal

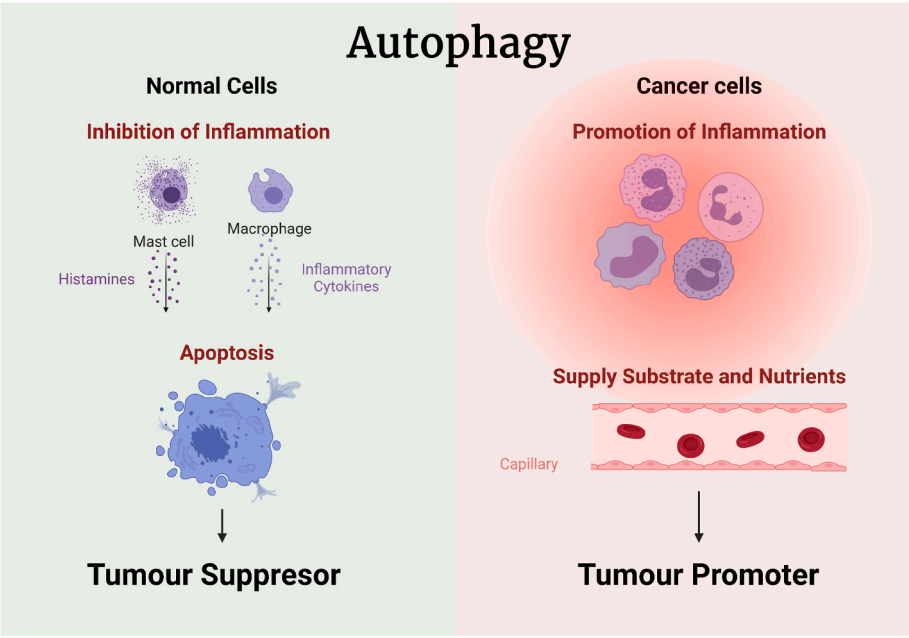

Fig. 5. Dual role of autophagy in cancer. Autophagy can be the tumor suppressor through the production and release of critical inflammatory cytokines and promotion of normal cells survival. In contrast, autophagy could be the tumor promoter by supplying substrates and nutrients as energy to cancerous cells under inadequate oxygen supply or any metabolic stress within the TME.

Table 3
Clinical trials involving hydroxychloroquine as combined therapies for malignancies (http://www.cancer.gov/clinicaltrials).

Indications	Status	Phase	Notes/Remarks	ClinicalTrials.gov identifier
B Cell chronic lymphocytic leukemia	Terminated	II	As single agent	NCT00771056
Bone metastases	Terminated	I	Combined with RT	NCT01417403
Cholangiocarcinoma	Recruiting	II	Combined with ABC294640	NCT03377179
Chronic myeloid leukemia	Unknown	II	Combined with Imatinib	NCT01227135
Colorectal carcinoma	Completed	I/II	Combined with Oxaliplatin and Leucovorin or 5-Fluorouracil and Bevacizumab	NCT01206530
	Completed	II	Combined with Vorinostat	NCT02316340
	Completed	II	Combined with Bevacizumab, Capecitabine and Oxaliplatin	NCT01006369
Gastrointestinal adenocarcinoma	Recruiting	I	Combined with Ulixertinib	NCT04145297
Glioblastoma multiforme	Completed	I/II	Combined with Temozolomide and RT	NCT00486603
Glioma	Completed	II	Combined with RT	NCT01602588
	Recruiting	I/II	Combined with Dabrafenib, Trametinib	NCT04201457
Hepatocellular carcinoma	Terminated	I/II	Combined with TACE	NCT02013778
	Recruiting	II	Combined with Sorafenib	NCT03037437
Melanoma	Completed	I	Combined with Vemurafenib	NCT01897116
	Completed	0	As single agent	NCT00962845
	Recruiting	I/II	Combined with Trametinib and Dabrafenib	NCT03754179
Metastatic breast cancer	Terminated	I/II	Combined with Ixabepilone	NCT00765765
	Terminated	I	As single agent	NCT02414776
	Recruiting	I/II	Combined with Letrozole, Palbociclib	NCT03774472
	Unknown	II	As single agent	NCT01292408
Metastatic NRAS Melanoma	Recruiting	0	Combined with Trametinib	NCT03979651
Multiple myeloma	Completed	I	Combined with Cyclophosphamide, Dexamethasone and Rapamycin	NCT01689987
	Completed	I/II	Combined with Bortezomib	NCT00568880
	Recruiting	I	Combined with Carfilzomib	NCT04163107
Non-small cell lung carcinoma	Terminated	I/II	Combined with Bevacizumab, Carboplatin and Paclitaxel	NCT00933803
	Terminated	II	Combined with Erlotinib	NCT01026844
	Active	II	Combined with Erlotinib	NCT00977470
	Unknown	I/II	Combined with Gefitinib	NCT00809237
	Terminated	I/II	Combined with Bevacizumab, Carboplatin and Paclitaxel	NCT00728845
	Completed	II	Combined with Bevacizumab, Carboplatin and Paclitaxel	NCT01649947
Pancreatic carcinoma	Completed	I/II	Combined with Gemcitabine	NCT01128296
	Completed	II	Combined with Abraxane and Gemcitabine	NCT01978184
	Active	I/II	Combined with Gemcitabine	NCT01506973
	Completed	II	As single agent	NCT01273805
	Recruiting	I	Combined with Trametinib	NCT03825289
	Recruiting	I	Combined with Binimetinib	NCT04132505
	Active	II	Combined with with Capecitabine and RT	NCT01494155
Prostate carcinoma	Unknown	II	As single agent	NCT00726596
	Terminated	II	Combined with Docetaxel	NCT00786682
	Terminated	II	Combined with Abiraterone and ABT-263	NCT01828476
Renal carcinoma	Terminated	I	As single agent	NCT01144169
	Completed	I/II	Combined with Everolimus	NCT01510119
	Completed	I/II	Combined with IL-2	NCT01550367
	Terminated	II	Combined with Rapamycin	NCT01842594
Refractory or recurrent ostesarcoma	Recruiting	I/II	Combined with Gemcitabine and Docetaxel	NCT03598595
Small cell lung cancer	Recruiting	II	Combined with Gemcitabine/carboplatin/Etoposide	NCT02722369
Soft tissue sarcoma	Terminated	II	Combined with Rapamycin	NCT01842594
Solid tumors	Completed	I	Combined with Temsirolimus	NCT00909831
	Active	I	Combined with Vorinostat	NCT01023737
	Active	I	Combined with MK2206(Akt inhibitor)	NCT01480154
	Recruiting	I	As single agent	NCT03015324
	Completed	I	Combined with Sorafenib	NCT01634893

Abbreviations: ABC294640*: [3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide]; RT: radiation therapy; ABT-263: Navitoclax; Akt: protein kinase B; MK2206: 8-(4-(1-Aminocyclobutyl)phenyl)-9-phenyl-8,9-dihydro-[1,2,4]triazolo[3,4-f][1,6]naphthyridin-3(2H)-one dihydrochloride; TACE: DescriptionTranscatheter arterial chemoembolization.*

formulation is also the first nanodrug approved by FDA for clinical uses [129]. The encapsulation of HCQ in liposome is an appealing approach that can increase its accumulation in tumors while ensuring a reduction in toxicity [111,113]. For example, Wang et al. [113] encapsulated HCQ in liposomes altogether with Salmonella VNP200009 for antitumor therapy. The encapsulation of HCQ causes the enhanced inactivation of lysosomal enzymes, which promotes the higher accumulation of Salmonella in tumor cells, thereby inhibiting their proliferation. To improve the tumor-targeting properties of the liposomes, cell-penetrating-peptide (CPP) that recognizes the overexpressed receptors on tumor cell surface including the ITGAV-ITGB3/(integrin $\alpha_v\beta_3$) and neuropilin-1 (NRP-1) can also be embodied onto the liposomes [114,115]. For instance, Wang and co-workers modify ZD6474 and HCQ co-loaded liposomes with R6dGR CPP to provide them the ability to recognize integrin $\alpha_v\beta_3$ and NRP-1 [114]. The modification thus renders the better blood-brain barrier (BBB) penetration, and successfully ensued the 4.4-folds and 3.8-folds higher uptake in bEnd.3 cells and C6 glioma cells as compared to the free HCQ. The higher accumulation eventually allowed the enhanced autophagy inhibition that leads to the increased sensitivity of the glioma cells towards the ZD6474 [114]. In another effort, a similar CPP, R8dGR peptide, has been deposited onto liposomes containing paclitaxel (PTX) and HCQ to improve their delivery to B16F10 melanoma cells [115]. The formulation has revealed the enhanced tumor-targeting capability *in vivo*, thereby yielding the higher autophagy inhibition (by HCQ), which leads to the increased antitumor efficacy by PTX [115].

In addition to receptor recognizing CPP, HCQ-loaded liposomes can also be functionalized with pH-responsivity to control its release behaviour. For example, Wang and colleagues modify the HCQ-containing liposomes with pH-responsive cell-penetrating TH peptide

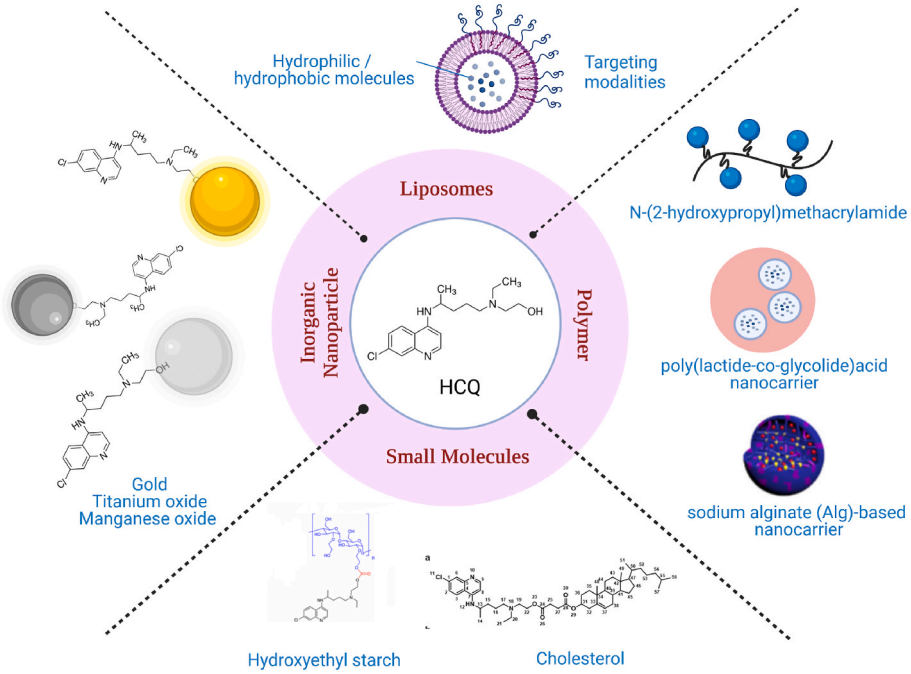

Fig. 6. Nanoassemblies/nanocarriers for HCQ include liposomes, inorganic nanoparticles, small molecular assemblies and polymeric assemblies.

[111]. Such incorporation enables the as-developed liposomes to bind to the integrin $\alpha_v\beta_3$ receptors on tumor surfaces and experience charge-reversal from negative (pH 7.4) to positive (pH 6.5) upon entering the tumor cells. It should be noticed that the greater extent of HCQ-induced lysosomal enzyme inhibition has been observed at pH 6.5 than at pH 7.4 (*in vitro* evaluation). This suggested the higher release of HCQ upon the pH-responsive charge-reversal. The pH-sensitive delivery has resulted in 35.68-folds and 15.16-folds increment in the intracellular concentration of HCQ when examined under *in vitro* and *in vivo* conditions, respectively. The formulation revealed an efficient blockage of autophagy flux in B16F10 tumor-bearing mice, which amplified the chemotherapeutic efficacy of DOX during the chemo and autophagy combined therapies [111].

Besides pH, other stimuli, namely thermal-, redox-, light-, and enzyme-sensitivity, have been developed for the preparation of smart liposomes [130–134]. These stimuli-responsive properties can also be explored further for the encapsulation and controlled delivery of HCQ. In fact, the utilization of GSH stimulant has shown promising controlled delivery performance for the HCQ encapsulated in other types of nanoassemblies. Furthermore, the inherently thermosensitive properties of liposomes also suggested the feasibility of designing a photothermal-, magnetic- or ultrasound controllable nanocarrier for the release of cargo. These examples will be introduced in further sections.

4.2. Inorganic nanoparticle

The uses of inorganic nanoassemblies in biomedical fields are drawing increasing attention recently due to their unique stimuli-responsivity and physicochemical properties [135]. Besides, for the nanoparticles that lack targeting properties, stimuli-sensitive ligands can also be incorporated to grant them the desired stimuli-responsive ability [136]. The possible active-control of inorganic nanoassemblies using both exogenous and endogenous stimuli has made them the desired nanotherapeutics carriers for tumor therapies [137]. The loading of bioactives or drugs into inorganic nanoparticles drug carriers may be done via the chemical conjugation with functional moieties [137]. The hydroxyl group on the HCQ structure is an exploitable functional group readily available for chemical interaction. One such example is the preparation of HCQ-conjugated gold nanoparticles. In the effort, gold nanoparticles are first PEGlated through thiol-gold bonding. Further, HCQ and siRNA are embodied onto the PEG-gold nanoparticles via Steglich esterification and thiol-gold crosslinking, respectively [117]. The deposited HCQ and siRNA tend to release from the nanocarrier at elevated glutathione (GSH) and dithiothreitol (DTT) conditions, which allowed the resultant siRNA-loaded HCQ-conjugated gold nanoparticles (GPHS) to achieve tumor targeting. This not only drives the improved endosomal escape activity (by HCQ) but also results in the effective down-regulation of luciferase in human cervix carcinoma cells [117]. Since the above-mentioned conjugation was carried out using the hydroxyl groups of HCQ, magnetic Fe_3O_4 nanoparticles may also hold potential as a template for HCQ-deposition. This is achievable by substituting the oxygen ions in Fe_3O_4 with the hydroxyl functional groups of HCQ during the Fe_3O_4 preparation. Such an idea is inspired by our earlier work that synthesized Fe_3O_4-nanocellulose nanocomposites via the same interaction (substitution of hydroxyl groups of nanocellulose against the oxygen ions in Fe_3O_4) [138]. Such innovation will be utmost useful for the generation of new inorganic nanoassemblies for tumor imaging and therapy owing to the extensive employment of magnetic nanoparticles as cancer therapeutics [137,139–142].

Besides the direct chemical conjugation route, HCQ can also be incorporated into inorganic nanoassemblies of hollow mesoporous structure. For instance, Feng et al. [92] developed an HCQ-loaded nanoarchitecture based on hollow mesoporous TiO_2 nanoparticles (HMTNPs) for antiautophagy-sonodynamic dual therapies. The HMTNPs are coated with cancer cell membrane (CCM) to obtain the active tumor homing ability to escape the macrophage phagocytosis. After tumor cell internalization, the ultrasound irradiation triggers the release of HCQ from the nanoassemblies, leading to the inhibition of autophagy and the increased sonodynamic therapy (SDT) efficacy to the cancer cells [92]. In addition, the authors also claimed the occurrence of vascular normalization by the HCQ, which relieved the hypoxic tumor environments, promoting the further enhancement of HMTNPs-mediated SDT (oxygen dependent). The outcome suggested the possible employment of HCQ for not only the autophagy inhibition but also the vascular remodeling in TME [92]. In another study, the HCQ is loaded into hyaluronic acid (HA)-coated hollow mesoporous Mn_2O_3

Table 4
Nanoassemblies employed for the encapsulation of HCQ for tumor-targeted therapies.

Nanoassemblies types	Nanoassemblies compositions	Loaded compounds	Targeted receptor/stimulus	Targeted therapies	Experimented tumor	Reference
Liposome	SPC, DSPE-PEG2000, cholesterol	HCQ, VNP200009		Autophagy inhibition (HCQ); bacterial cancer therapy (VNP200009)	B16F10 Melanoma (*in vivo*)	[113]
	SPC, DSPE-PEG2000, cholesterol, R6dGR	HCQ, ZD6474	Integrin $\alpha_v\beta_3$, NRP-1	Autophagy inhibition (HCQ); chemotherapy (ZD6474)	C6 Glioma (*in vivo*); bEnd.3 cells (*in vitro*)	[114]
	SPC, DSPE-PEG2000, cholesterol, R8dGR	HCQ, PTX	Integrin $\alpha_v\beta_3$, NRP-1	Autophagy inhibition (HCQ); chemotherapy (PTX)	B16F10 Melanoma (*in vivo*)	[115]
	SPC, DSPE-PEG2000, cholesterol, TH-RGD	HCQ, DOX	Integrin $\alpha_v\beta_3$, pH	Autophagy inhibition, stroma fibrosis inhibition (HCQ); chemotherapy (PTX)	BXPC-3 human pancreatic adenocarcinoma cells (*in vivo*)	[116]
	SPC, DSPE-PEG2000, cholesterol, TH-RGD	HCQ, DOX	Integrin $\alpha_v\beta_3$, pH	Autophagy inhibition (HCQ), chemotherapy (DOX)	B16F10 Melanoma (*in vivo*)	[111]
Inorganic nanoparticle	SH-PEG2000, gold	HCQ, siRNA		Autophagy inhibition (HCQ); gene silencing (siRNA)	HeLa-luc human cervix carcinoma cells expressing luciderase (*in vitro*)	[117]
	TiO_2, CCM,	HCQ	CD44, CD47, CD176, galectin-3, E-cadherin	Autophagy inhibition and vascular normalization (HCQ); SDT (TiO_2)	MCF-7 Human breast cancer cell (*in vivo*)	[92]
	Mn_2O_3, HA	HCQ	CD44, pH, GSH	Enhanced autophagy inhibition (Mn^{2+} + HCQ); MRI (Mn^{2+})	4T1 Breast cancer cell (*in vivo*)	[118]
Small molecule	HCQ, HES	HCQ-HES	CXCR4	Autophagy inhibition (HCQ-HES)	AsPC-1, MiaPaca-1, MiaPaca-2 Pancreatic cancer cell line *(in vitro)*	[119]
	Cholesterol, HCQ	Chol-HCQ		Bleomycin-induced pulmonary fibrosis (Chol-HCQ)	Lung fibroblast	[120]
Polymer	HPMA, HCQ	pHCQ	CVCR4	Antimestastatic therapies (pHCQ)	human T lymphoblastoid cells (Jurkat), human Burkitt's lymphoma cells (Raji) (*in vitro*)	[121]
	HPMA, HCQ	pHCQ	CVCR4/CVCL12	Antimestastatic therapies (pHCQ)	U2OS Human epithelial osteosarcoma, HepG2 Human hepatocellular carcinoma, A549 Lung cancer cell (*in vitro*); 4T1 Breast cancer cell (*in vivo*)	[122]
	PLGA	HCQ, OVA		Autophagy inhibition (HCQ); immune system boosting (OVA)	E.G7-OVA mouse thymic lymphoma cells (*in vivo*)	[123]
	PDMAEMA, MACQ	HCQ, miRNA	pH, temperature	Autophagy inhibition (HCQ); gene silencing (miRNA)	MDA-MB-231 human mammary gland adenocarcinoma cell (*in vitro*)	[124]
	PLA-b-PEG-COOH, anti-CD20	HCQ, CLB	Neoplastic B-cells	Autophagy inhibition (HCQ); chemotherapy (CLB)	MEC1 chronic lymphocytic leukemia cell (*in vivo*)	[125]
	CA4, Fe^{3+}, Alg	HCQ	pH, GSH	Vascular disruption (CA4); autophagy inhibition (HCQ); chemodynamic therapy (Fe^{2+})	A549 lung cancer cell (*in vivo*)	[126]

nanoparticles (HA-Mn_2O_3/HCQ) for cancer theranostics [118]. With HA as the tumor-targeting moiety, the Mn_2O_3-based nanoassemblies bind to the CD44 receptor to internalize into the 4T1 tumor cells. The Mn_2O_3 then rapidly degrade into Mn^{2+} in the acidic and elevated GSH intracellular environments, thereby activating the synergistic autophagy inhibition (by HCQ + Mn^{2+}) and MRI simultaneously [118]. The investigations showed effective imaging and repression of the growth of 4T1 tumor cells in mouse model.

Despite the limited examples showing the nanoencapsulation and controlled delivery of HCQ in inorganic nanostructures, the available evidence suggested the possible programming of nanoassemblies that are manipulable via either exogenously or endogenously. The involvement of these successes to the other nanoassembly systems (e.g., liposomes) will be beneficial to the overall development of the nanoformulated HCQ for controlled tumor therapy.

4.3. small molecules

Small molecules-based nanoassemblies generally refer to those consisting of organic compounds of low molecular weight (<900 Da) [143]. Examples of small molecular compounds include many of the existing clinical drugs that are naturally hydrophobic. Nevertheless, the hydrophobic properties also reflect the low solubility and bioavailability of these drugs, which may retard their delivery efficiency upon utilization [144]. The controlled assembly of these small molecular drugs appears to be a useful tactic to enhance the aqueous solubility and bioavailability of these compounds simultaneously. For example, Sleightholm et al. [119] prepared HCQ-conjugated hydroxyethyl starch (HES) nanoparticles with excellent antitumor properties. The conjugation is performed via the carbonyldiimidazole coupling between the HES and the HCQ (both contain hydroxyl groups for reaction). The formulation showed incredible stability across pH 5 to 7.4 and only underwent hydrolytic cleavage resulting in the release of HCQ at pH 2. This suggested the enhanced stability of the HCQ-modified HES (HCQ-HES) nanoparticles as a drug nanoassembly for tumor therapy [119]. Additionally, the presence of secondary alkyl-aryl amine in the HCQ structure also promotes the CXCR4 receptor binding [145], which ensured the tumor-targeting ability of the HCQ-HES nanoparticles. The HCQ-HES nanoparticles revealed improved autophagy inhibition than the free HCQ, leading to the better inhibition of the migration and invasion of pancreatic cancer cells [119]. In another study, Liu et al. [120] prepared cholesterol-HCQ (Chol-HCQ) conjugates for the treatment of bleomycin-induced pulmonary fibrosis. The Chol-HCQ is constructed via a two-step approach, starting with the reaction between the cholesterol and succinic anhydride to provide the reactive hydroxyl groups to the cholesterol, followed by reaction with HCQ to synthesize Chol-HCQ. It demonstrated the significant inhibition of the extracellular signal-regulated kinase 1/2 (ERK1/2) and nuclear factor kappa beta (NF-κβ) in the lung fibroblasts *in vitro* as compared to the free HCQ [120]. The authors then performed the *in vivo* investigation on bleomycin-treated mouse model after the further encapsulation of Chol-HCQ in liposomes. The *in vivo* evaluation showed better

suppression of the pulmonary fibrosis by the Chol-HCQ-loaded liposomes as compared to HCQ-loaded liposomes, suggesting the capability of the cholesterol to amplify the anti-fibrosis characteristics of the HCQ [120].

Although the small molecular assembly exhibited supreme chemotherapeutics efficacy over the individual drugs, it is undeniable that the HCQ in these formulations still exposed to the external microenvironment. This indicated the possible premature release, hydrolytic degradation and toxicity to healthy tissues. Thus, the encapsulation of the nanoformulated HCQ into larger nanostructures may be more promising to provide protection over the TME and indiscriminate attacks to normal tissues.

4.4. Polymer

Polymer has imparted remarkable contributions in the pharmaceutical industry for drug encapsulation and targeted delivery due to several advantages, including the improved safety, control and delivery performance of existing drugs [122]. The use of polymers can range from the stimuli-responsive moieties that aid in the release of encapsulated cargos to the ligands with the ability to target the designated receptors [146,147]. Moreover, polymer differs from small molecules in terms of their larger molecular mass, various unique physicochemical properties and functional groups available for self-assembly despite their similar molecular element [137]. Polymeric nanoassemblies have been extensively presented for HCQ encapsulation (as listed Table 4), which can take the forms of drug-polymer conjugate, polymersomes and hydrogels. As an example, Yu and others co-polymerize HCQ with N-(2-hydroxypropyl)methacrylamide (HPMA) using azobis(isobutyronitrile) (AIBN) as the initiator to forms polymeric HCQ (pHCQ) for cancer treatment [121,122]. The study revealed that pHCQ not only allowed the anti-autophagy effect but also inhibited the cancer cell migration and invasion due to the CXCR4/CXCL12 silencing. The outcomes of the study showed the supreme antimetastatic activity of pHCQ with lower cytotoxicity over a series of tumor cells compared to bare HCQ [122].

In another contribution, Liu et al. [123] co-loaded HCQ and ovalbumin (OVA) in poly(lactide-*co*-glycolide)-acid (PLGA)-based nanocarriers for antitumor immune response enhancement. The presence of HCQ induced the higher OVA escape from lysosomes, realizing the enhanced OVA effects as compared to the controls. Additionally, the *in vivo* experiments also suggested the higher $CD8^+$ T-cell count and initiation of Th1-type response, leading to tumor cell apoptosis and prevention of tumor progression [123]. This demonstrated the promise of co-delivering HCQ and antigen for combined antiautophagy and immunotherapies.

Nanogel is also a polymer assembly product that exhibits great aqueous dispersibility, physiological stability and structural plasticity [137,148]. Zhang and colleagues have expressed the first evidence of the nanoencapsulation of HCQ in sodium alginate (Alg)-based smart nanogel for multiple tumor therapies [126]. The nanogel consists of an antiangiogenic compound combretastatin A4 (CA4), Alg body, Ferrum ions as the stimuli-responsive ligands and HCQ as the antiautophagy drug. The Alg and CA4 first undergo Yamaguchi reaction to create an ester bonding between the carboxyl groups of Alg and hydroxyl groups of CA4, yielding Alg-CA4. The Fe^{3+} is then utilized as a cross-linker to fabricate the CA4-FeAlg nanogel, followed by the HCQ loading via the swelling of CA4-FeAlg nanogel in HCQ solution [126]. During the antitumor test in the A549 tumor-bearing mice model, the CA4 is first released around the tumor site to disrupt the tumor vessel, thereby depleting the oxygen and nutrient supply to the tumor cells. The Fe^{3+} then reduced to Fe^{2+} owing to the acidic pH and high GSH level in the TME, further disintegrating the nanogel, which released the HCQ for autophagy inhibition. The antiautophagy process then ensured the presence of Fe^{2+} in tumor cells for initiating the Fenton reaction with H_2O_2 to generate hydroxyl radicals for tumor elimination. The as-prepared nanogel displayed 60% reduction in tumor volume (over the initial tumor volume, V/V_o) against A549 tumor-bearing nude mice *in vivo*. [126] Despite the noticed effectivity of the newly developed nanoassemblies for tumor therapy, the researches on the nanoencapsulation and targeted-delivery of HCQ is still considered limited. More attempts should be committed to the design of the new HCQ-nanocarrier as these will be essential for the design of new generation of smart nanomedicine for multiple combinational tumor therapies.

5. Conclusions and future perspectives

Despite its classical indication as an antimalarial drug, HCQ possesses a diversity of clinical and biological properties. Evidence suggesting that HCQ is less toxic than its parent compound CQ has led to its increased use [149]. It has been evaluated in more than 30 infectious and non-infectious disorders more than 50 years. Among the activities reported in the literature, the most mentioned indication is the treatment of autoimmune diseases such as RA and SLE, where the drug has been established as an alternative therapy. Although HCQ has been used for many years, their exact mechanisms of action underlying the drug being used in various kind diseases or disorders are still not well understood. Accumulating evidence suggests that HCQ has multiple effects on several cellular pathways, such as autophagy inhibition and interfering certain signaling pathways. Reports also reveal the effectiveness of HCQ in antimicrobial, antineoplastic, multiple sclerosis, treatment of infection by HIV and in different conditions associated with existing rheumatic conditions, such as improved insulin sensitivity, reduction of lipid levels, ability to reduce thromboembolic events, and reduce the risk of cardiovascular diseases. The interesting multifaceted actions of HCQ against various diseases, particularly in cancer has made the drug an attractive target for cancer therapies.

At the cellular level, a few mechanisms have been described that account for the antitumor effects of HCQ. In particular, inhibition of autophagy prevents immune activation of different cell types in TME, which inhibits cytokine production and modulates the immune response. Targeting autophagy for potential and promising cancer treatment is gaining attention as TME is very important to tumor growth, metastasis, and therapy resistance, although the mechanism of autophagy interfaced with TME still remains unclear and needs to be explored. Drug-drug interactions represent the most limiting factor during the clinical management of any disease, in particular when several drugs are co-administered to treat the same disease. Certain drug interactions (such as interactions with tamoxifen, glycosides, methotrexate and ciclosporin) in combined cancer therapies can influence the pharmacokinetics of HCQ and hence require consideration [56]. It might be prudent to develop a consensus based on preclinical data as to which types of cancer and which class or classes of drugs used in standard regimens is most appropriate with least drug-drug interactions for testing in the context of clinical trials of HCQ or other modulators of autophagy. Taken together, HCQ is new promise of old drug for effective cancer therapies. Indeed, various tumor-associated animal models as well as testings in humans have confirmed that autophagy inhibition at early or late stage leads to prevention of the autophagosome formation, enhancing chemosensitivity and promoting tumor regression. However, the use of autophagy-targeted therapy still needs to be cautious due to its dual roles in tumor growth progress.

It is uncertain whether HCQ actually inhibits autophagy in human tumors and whether the extent of inhibition would be sufficient to alter chemotherapy or radiotherapy sensitivity. Additional mechanistic studies in preclinical models are clearly required to understand the role of autophagy inhibition in the context of cancer therapies. Besides, it has some dose-sensitive effects, and it is necessary that physicians using HCQ to clinical treatment should consider the toxicity of HCQ retinopathy that threatens human health. Current evidences also reinforce the need for special care in the treatment management in long term with HCQ, since the ocular and cutaneous adverse effects, possible phototoxic

reactions, can cause irreversible damage. Additionally, some conventional therapeutics also suffer from poor solubility, bioavailability and controlled release mechanisms. In short, the effect of HCQ explored in the current clinical trials for various cancers is still unsatisfactory. To further investigate the roles of HCQ in cancer therapy, a number of molecular modifications, such as the nanocarrier for HCQ, have been used with the aims of improving pharmacokinetic and pharmacodynamic properties, reducing undesirable side effects, costs and drug sensitivities [45]. It appears that novel nanoplatforms overcome these obstacles and have led to the design of a theragnostic-controlled drug release system with high solubility and active targeting and stimuli-responsive potentials.

To date, the use of nanoassemblies in tumor-targeted therapies has obtained increasing attention, leading to extensive research on more new and innovative drug carriers [136,137,140,150,151]. The nanoencapsulation or nanoconjugation of HCQ with nanoassemblies appears to be a promising method for reducing the toxicity and improving the antitumor efficacy of HCQ. Various nanocarrier, namely liposomal, inorganic nanoparticle, small molecular and polymeric nanoassembly systems have been explored and showed significant antitumor effects in various cell lines and mice models. However, to the best of authors' knowledge, the HCQ-based nanoassembly system remained poorly explored. Additionally, some of the innovative nanoassemblies system with HCQ incorporation did not focus on antitumor therapy. For example, the niosome, an alternative phospholipid vesicle constructed from cholesterol and non-ionic surfactants were also reported as an excellent reservoir for HCQ [152]. Furthermore, many of the newly invented nanoassembly systems, which have shown the successful encapsulation/conjugation of chemotherapy drugs (DOX and PTX), can also be attempted for HCQ encapsulation/deposition to prepare a new nanomedicine for combinational tumor therapies [153–160]. It should be noted that the HCQ is applicable for immune enhancement via the remodeling of tumor-associated macrophage (TAM) from immune-suppressing M2-like macrophage to tumor-suppressing M1-like macrophages [161]. These properties can be evaluated in the future using the nanoformulated HCQ for site-specific macrophages re-engineering, similar to those performed using the polymeric dynamic nanoparticles [162,163]. Nevertheless, majority of the potential nanocarriers developed for enhanced tumor therapy are presented with a homogeneous cell population using *in vivo* mice model. Results deficiency would be expected upon employment in heterogeneous clinical tumors in the next stage, similarly to the clinical trials using free HCQ [164]. Thus, to select the desired remedies for patients, more biomimetic models, such as patient-derived xenografts and genetically engineered mice models, should be performed to help address the possible challenges of tumor heterogeneity [165]. The successful overcoming of tumor heterogeneity would likely to provide vital boost to the development of the advanced nanotherapeutics, which hold promises for clinical translation in the near future.

Credit author statement

L.E.L., C.K.K. Writing, Review & Editing.; L.E.L. Supervision, Conceptualization, Writing, Review & Editing.; W.-H.Y., S.P.S., W.S.S., L.C.M., A.S.L.-F., W.-L.L., S.H.G., S.K.C, Y.W., B.T.T., K.-Y.K., Y.S.O., D. K.C., K.D., S.K.S. Review & Editing.; B.-H.G. Supervision, Writing, Review & Editing, Project administration, Conceptualization.

Notes

The authors declare no conflict of interest.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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