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Effects of chloroquine and hydroxychloroquine on bone health (Review)

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Abstract. Chloroquine (CQ) and hydroxychloroquine (HCQ), which were initially used to treat malaria, are now also used to treat autoimmune and inflammatory diseases, which have gained notoriety during the coronavirus-19 pandemic. The emerging uses of CQ and HCQ in cancer therapy, metabolic syndrome and bone disorders highlight their broad clinical potential. Patients with autoimmune and inflammatory conditions have a higher risk of suboptimal bone health because of chronic inflammation, immune dysregulation and medication use. In the present review, the use of CQ and HCQ in bone research was explored, particularly in terms of their effectiveness and mechanism in modulating bone homeostasis. CQ and HCQ inhibit osteoblastic activity by suppressing autophagy, inducing oxidative stress and promoting osteoblast apoptosis. CQ suppresses osteoclastic activity by blocking the receptor activator of nuclear factor κ-B/receptor activator of nuclear factor κ-B ligand interaction, autophagy and inflammation. HCQ inhibits osteoclastogenesis by increasing the expression levels of osteoprotegerin, inducing osteoclast apoptosis and reducing cytokines without affecting autophagy. With regard to the molecular machineries, CQ and HCQ inhibit bone formation and bone resorption. Variations in dose, frequency and duration of CQ and HCQ treatment result in heterogenous outcomes. Further research is necessary to clarify the net effects of CQ and HCQ on bone through studies specifically designed to explore their direct impact as the primary objective. The use of these medications is broadening particularly in patients with autoimmune diseases who are at risk of skeletal disorders. However, their safety profiles, adverse effects and contraindications must be carefully monitored when administered for long-term use and in combination.

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Abbreviations: ADO2, autosomal dominant osteopetrosis type II mutation; ALP, alkaline phosphatase; AMPK, 5'AMP-activated protein kinase; Atg, autophagy-related protein; BMD, bone mineral density; BMM, bone marrow monocyte/macrophage; BMSC, bone marrow mesenchymal stem cell; BV/TV, bone volume/tissue volume; CAT, catalase; c-fms, macrophage colony-stimulating factor receptor; c-Fos, Fos proto-oncogene; CQ, chloroquine; CTSK, cathepsin K; CTX, cross-linked C-telopeptide of type 1 collagen; DCQ, desethylchloroquine; GPx, glutathione peroxidase; HCQ, hydroxychloroquine; HR, hazard ratio; i.p., intraperitoneal; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; Nbr1, neighbour of Brca1 gene; NFATc1, nuclear factor of activated T-cells cytoplasmic 1; NO, nitric oxide; Nox4, nicotinamide adenine dinucleotide phosphate oxidase 4; OCN, osteocalcin; Oc.N, osteoclast number; OPG, osteoprotegerin; OPN, osteopontin; PBMC, peripheral blood mononuclear cell; P1NP, propeptide of type I procollagen; p62/SQSTM1, sequestosome-1; RANK, receptor activator of nuclear factor κ-B; RANKL, receptor activator of nuclear factor κ-B ligand; ROS, reactive oxygen species; Runx-2, Runt-related transcription factor 2; SLE, systemic lupus erythematosus; SOD, superoxide dismutase; Tb.N, trabecular number; Tb.Sp, trabecular separation; TRAF, tumour necrosis factor receptor-associated factor; TRAP, tartrate-resistant acid phosphatase; β-CTx, β C-terminal telopeptide; ULK1, Unc-51-like kinases 1; Vps, vacuolar protein sorting; Th17, T helper 17; RNS, reactive nitrogen species

Key words: BMD, bone microstructure, CQ, fracture, HCQ, osteoporosis

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1. Introduction

Bone is a dynamic organ constantly undergoing remodelling, which consists of two coordinated processes: Bone resorption by osteoclasts and bone formation by osteoblasts. The coupling imbalance between bone remodelling, indicated by

low bone-forming osteoblast activity and high bone-resorbing osteoclast activity, is the basic mechanism of osteoporosis. Osteoporosis is a bone disorder in which bones become weak, brittle and fragile, leading to increased susceptibility to fracture (1). A direct association of autoimmune diseases [such as rheumatoid arthritis and systemic lupus erythematosus (SLE)] and inflammatory diseases (such as systemic sclerosis and sarcoidosis) with impaired bone health has been reported, which can be explained by inflammation, immune dysregulation and the use of medications (2-4). Chronic inflammatory responses promote osteoclastic activity and accelerate bone breakdown, resulting in bone loss (5). T helper 17 (Th17) cells that produce IL-17 are overactive in various autoimmune and inflammatory disorders, which induces the receptor activator of nuclear factor κ-B ligand (RANKL) and inhibits osteoprotegerin (OPG) production by osteoblasts (6). The long-term use of glucocorticoids and methotrexate adversely affect bone metabolism and increase the risk of fractures (7,8).

Chloroquine (CQ) and hydroxychloroquine (HCQ) are medications used to prevent and treat malaria caused by *Plasmodium* parasites. CQ and HCQ interfere with haemoglobin proteolysis in the parasite causing the accumulation of toxic byproducts to kill the parasite (9). Apart from their anti-plasmodial property, CQ and HCQ have diverse pharmacological effects on other medical conditions, including autoimmune diseases (10), cancer (11), viral infections (12), metabolic syndrome (9) and skeletal disorders (13,14), because of their anti-inflammatory and immunomodulatory properties. Chronic therapy with CQ or HCQ is necessary to manage autoimmune diseases (10). Therefore, investigating the effects and underlying molecular mechanism of CQ or HCQ on the skeleton could yield valuable information to prevent osteoporosis with minimal side effects.

The present review aims to summarize the role of CQ and HCQ in protecting the bones from becoming porous. It also highlights the underlying molecular mechanisms that govern the action of CQ and HCQ. The present review may aid researchers and interprofessional healthcare teams in recognizing the skeletal-protecting potential of CQ and HCQ, and their mechanisms of action.

2. Evidence acquisition

The research question set for the present review was as follows: 'What are the effects of CQ and HCQ on bone health and their underlying mechanism of actions?'. To identify relevant studies to be included in the present review, a comprehensive literature search was caried out using two electronic databases [PubMed (https://pubmed.ncbi.nlm.nih.gov/) and Web of Science (https://www.webofknowledge.com/)] in June 2024 using the search string '(chloroquine OR hydroxychloroquine) AND (bone OR osteoporosis OR fracture OR osteoblast OR osteoclast OR osteocyte)'. All available primary studies examining the effects of CQ or HCQ on bone health from the inception of the databases were considered. Articles without relevant results or primary data (including reviews, letters to the editor, perspectives, books and book chapters) were not considered. Conference abstracts and proceedings were excluded because of the preliminary nature of the data and the potential of data duplication from full-text articles. Articles not written in English were also excluded.

The literature search using the two electronic databases identified 1,383 records (PubMed, 755; Web of Science, 628). Duplicates (n=442) were removed. Preliminary screening of the title and abstract identified reviews (n=115), commentary (n=1), preprint (articles under review; n=1), conference proceedings (n=2), non-English articles (n=56), irrelevant articles (n=705) and retracted articles (n=2) to be excluded. Full-text articles were screened for eligibility based on the inclusion and exclusion criteria. All original research articles reporting bone-related changes and understanding of the mechanisms resulting from the monotherapy of CQ and HCQ or combined therapy with other drugs were included. Three articles were excluded after screening of full-text articles because they did not report the direct effects of CQ and HCQ on bone parameters as study outcomes. A total of 56 articles adhering to the inclusion criteria were included in the present review (Fig. 1).

3. Effects of CQ and HCQ on bone health in humans

Accumulating evidence has revealed heterogenous outcomes for the effects of CQ and HCQ on bone health, whereby positive, negative and negligible effects have been observed (Table I). The literature search using the search string identified an early small-scale study investigating the effects of CQ on serum bone markers in patients with rheumatoid arthritis and seronegative spondyloarthropathies. The level of serum osteocalcin (OCN) was increased, while the level of alkaline phosphatase (ALP) remained unchanged after CQ therapy at a dose of 250 mg/day (15). Previous studies involving humans have shifted to elucidate the effects of HCQ treatment on bone health. In a retrospective nationwide cohort study involving patients with rheumatoid arthritis with and without new-onset cardiovascular disease (n=2,534), who were aged ≥40 years, the adjusted hazard ratio (HR, 0.12; 95% CI, 0.03-0.47) of developing osteoporotic vertebral fracture was lower in patients receiving HCQ in a Taiwanese cohort (13). In Japan, a cross-sectional study was conducted on outpatients with SLE (n=246) to examine the association of HCQ use with T-score and vertebral fractures. HCQ use was associated with high lumbar (coefficient, 0.44; 95% CI, 0.077-0.81) and femoral (coefficient, 0.33; 95% CI, 0.020-0.63) T-scores. In addition, HCQ use was associated with a lower incidence of vertebral fractures (odds ratio, 0.098; 95% CI, 0.013-0.73) (14).

Both *et al* (16) recruited patients with rheumatoid arthritis (n=63) aged ≥18 years, who were receiving HCQ as treatment. The levels of a serum bone resorption marker [β C-terminal telopeptide (β-CTx)] and an inflammatory marker (CRP) at baseline and after 6 months were measured. Blood samples were collected at random moments throughout the day without fasting. HCQ treatment for 6 months decreased the levels of β-CTx after adjusted for the decrease in serum CRP, indicating that HCQ had a direct effect on bone resorption, and the decrease in β-CTx was independent of the inflammatory response reduction (16). Yu *et al* (17) studied the effects of combined therapy of disease-modifying anti-rheumatic drugs (iguratimod, methotrexate and HCQ) on bone

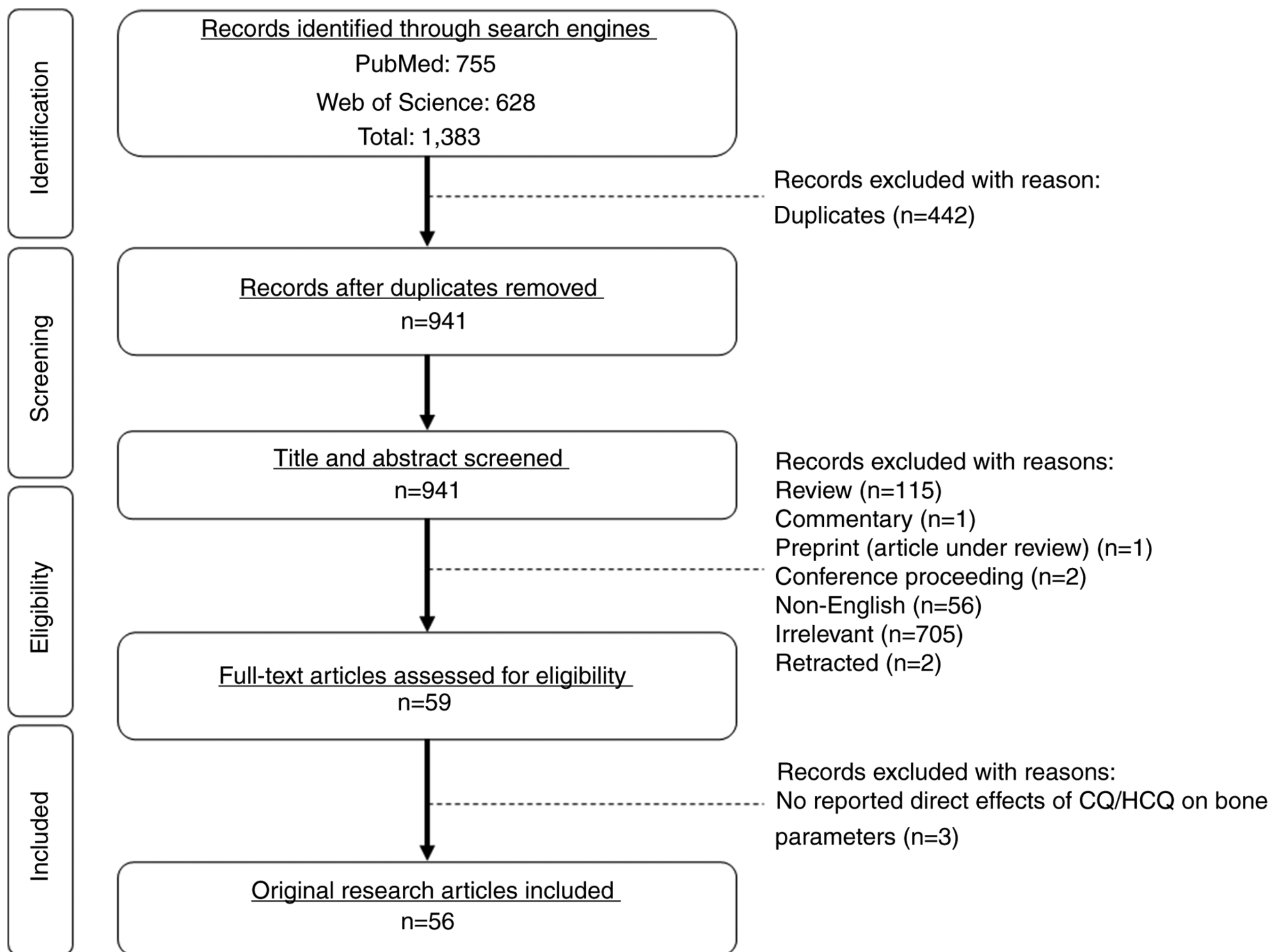

Figure 1. Diagram displaying the identification, screening and assessment of publications associated with the effects of CQ and HCQ on bone health. CQ, chloroquine; HCQ, hydroxychloroquine.

mineral density (BMD) in patients with rheumatoid arthritis (n=76; age, 59.05±10.95 years). BMD at the lumbar spine (L1-L4), left femoral neck and left total hip increased after 48 weeks of treatment. The combined therapy also reduced rheumatoid factor, CRP, the erythrocyte sedimentation rate and anti-cyclic citrullinated peptide (17).

However, two human studies reported negative effects of HCQ on bone health. A recent study by Park *et al* (18) found that patients with juvenile-onset SLE and low BMD had a higher cumulative HCQ dose (10.10 g/kg), whereas those with non-low BMD had a lower cumulative HCQ dose (4.64 g/kg). In addition, the cumulative HCQ dose was negatively associated with the lumbar spine BMD Z-score in patients with juvenile-onset SLE (18). Heidari *et al* (19) revealed that the BMD of the femoral neck and lumbar spine in patients with rheumatoid arthritis (n=19; age, 54.5±7.7 years) decreased after treatment with low-dose methotrexate (≤15 mg/week) alone or in combination with HCQ or sulfasalazine and prednisolone (5 mg) (19).

One study by Carbone *et al* (20) revealed the negligible effects of HCQ on bone health. A total of 363 women with rheumatoid arthritis aged 50-79 years who were treated with HCQ alone or combination therapy of HCQ and methotrexate were enrolled. Fracture incidence following HCQ use was estimated over a median follow-up length of 6.46 years. No significant association was identified between HCQ use and fracture incidence (HR, 1.0; 95% CI, 0.7-1.5). A similar relationship was noted between HCQ and methotrexate use and fracture incidence (HR, 0.9; 95% CI, 0.5-1.6) (20).

Collectively, the findings from the majority of the aforementioned studies indicated the potential protective effects of CQ and HCQ in improving bone health (13-17). One study reported that HCQ alone had an inhibitory effect on bone resorption, which was not influenced by the reduction of inflammation (16). On the contrary, another study indicated that inflammatory indicators decreased in response to combination drug therapy consisting of iguratimod, methotrexate and HCQ (17). Therefore, the suppression of the inflammatory reaction observed by Yu *et al* (17) may be at least partially attributed to iguratimod and/or methotrexate. Several methodological limitations (such as single-arm studies, small-scale samples, short study duration and lack of standardisation) may lead to fallacious conclusions, as well as less accurate and reliable results. The dose of HCQ that was administered was also not mentioned in some of the aforementioned studies (13,14,16,20). Two studies reported negative outcomes (18,19), which may be attributed to the small sample size and lack of control group.

Table I. Studies investigating the effects of CQ on bone health in humans.

First author/s, year	Study population	Treatment	Findings	(Refs.)
Ekenstam *et al*, 1986	Patients with rheumatoid arthritis (n=36; aged 20-69 years) and seronegative spondyloarthropathies (n=23; aged 26-67 years)	CQ (250 mg/day)	Serum OCN was markedly increased after 3 and 6 months, but ALP was unchanged after 3 and 9 months.	(15)
Hong *et al*, 2019	Patients with rheumatoid arthritis and new-onset CVD or without CVD (n=2,534; aged ≥40 years)	HCQ (dose unspecified)	Lower risk of osteoporotic vertebral fracture in patients with rheumatoid arthritis who were receiving HCQ (HR, 0.12; 95% CI, 0.03-0.47).	(13)
Nakajima *et al*, 2023	Patients with SLE (n=246; median age, 47 years)	HCQ (dose unspecified)	The use of HCQ was associated with high lumbar (coefficient, 0.44; 95% CI, 0.077-0.81) and femoral (coefficient, 0.33; 95% CI, 0.020-0.63) T-scores. The use of HCQ was associated with a lower incidence of vertebral fractures (OR, 0.098; 95% CI, 0.013-0.73).	(14)
Both *et al*, 2018	Patients with rheumatoid arthritis (n=63; aged ≥18 years)	HCQ (dose unspecified)	Serum β-CTx was lowered after 6 months.	(16)
Yu *et al*, 2021	Patients with rheumatoid arthritis (n=76; aged 59.05±10.95 years)	Iguratimod (25 mg; twice a day) + methotrexate (10 mg; once per week) + HCQ (200 mg; twice a day) for 48 weeks	BMD at L1-L4, left femoral neck and left total hip were increased. Rheumatoid factor, CRP, ESR and anti-CCP were reduced.	(17)
Park *et al*, 2024	Patients with juvenile-onset SLE (n=29; aged 11.5-14.8 years)	Cumulative HCQ (4.64 or 10.1 g/kg)	Patients with a higher cumulative HCQ dose exhibited low BMD. A higher cumulative HCQ dose was associated with a low lumbar spine BMD Z-score.	(18)
Heidari *et al*, 2012	Patients with rheumatoid arthritis (n=19; aged 54.5±7.7 years)	Low-dose methotrexate (≥15 mg/week) alone or a combination of HCQ and/or sulfasalazine and prednisolone (5 mg)	BMD of the femoral neck and lumbar spine decreased.	(19)
Carbone *et al*, 2020	Women with rheumatoid arthritis (n=363; aged 50-79 years)	HCQ (dose unspecified)	No significant association between HCQ use and fracture incidence (HR, 1.0; 95% CI, 0.7-1.5). No significant association between combination therapy of HCQ and methotrexate and fracture incidence (HR, 0.9; 95% CI, 0.5-1.6).	(20)

ALP, alkaline phosphatase; anti-CCP, anti-cyclic citrullinated peptide; BMD, bone mineral density; CQ, chloroquine; CVD, cardiovascular disease; ESR, erythrocyte sedimentation rate; HCQ, hydroxychloroquine; HR, hazard ratio; OCN, osteocalcin; OR, odds ratio; SLE, systemic lupus erythematosus; β-CTx, β C-terminal telopeptide.

4. *In vivo* studies: Effects of CQ and HCQ on bone health in animals

The effects of CQ and HCQ on bone health have been examined using healthy animals and animal models, including osteoporotic, knockdown, arthritic, fractured and osteotomy models (Table II). An early study examined the quantitative distribution of CQ radioactivity in the bone tissues of animals. Adult and 8-day-old Sprague-Dawley rats, as well as 1-day-old leghorn chickens received intraperitoneal (i.p.) injections of [3-^{14}C]CQ. The radioactivity of CQ recovered in the bone was 11.6%, indicating the high affinity of CQ for bone (21).

Dexamethasone is a glucocorticoid medication used to relieve inflammation (including swelling, heat, redness and pain) that causes secondary osteoporosis (22). The continuous administration of dexamethasone (2 mg/kg) for 35 days resulted in lower ALP activity and higher tartrate-resistant acid phosphatase (TRAP) activity in mice. The i.p. injection of CQ (50 mg/kg) for 7 days (between days 28 and 35) caused a reduction in TRAP activity without altering ALP activity (23). TRAP is a key enzyme produced by osteoclasts to break down bone matrix proteins during bone resorption. Thus, lower TRAP activity suggests reduced osteoclast activity and bone resorption. In another dexamethasone-induced osteoporotic mouse model, i.p. injection of dexamethasone (1 mg/kg) for 8 weeks reduced the bone volume/tissue volume (BV/TV), trabecular number (Tb.N), osteoblast number and osteoblast surface, and increased the osteoclast number (Oc.N). The injection of CQ (2 mg/kg; i.p.) every 2 days mitigated dexamethasone-challenged bone loss (increased BV/TV and Tb.N) by reducing Oc.N without affecting the parameters linked to osteoblasts and bone formation (24).

Bilateral ovariectomy removes the ovaries and eliminates the secretion of oestrogen in animals, representing a preclinical model for postmenopausal osteoporosis (25). The administration of CQ (10 mg/kg; i.p.) twice a week for 8 weeks reduced cross-linked C-telopeptide of type 1 collagen (CTX; a bone resorption marker), but did not change the level of propeptide of type I procollagen (P1NP; a bone formation marker) in ovariectomised rats (26). Lin *et al* (24) investigated the effects of CQ (2 mg/kg; i.p.) administered every 2 days for 8 weeks on bone parameters using ovariectomised mice. Treatment with CQ resulted in a reduction in Oc.N, but no change was observed in osteoblast counts in ovariectomy-induced bone loss (24). In another study, CQ prevented ovariectomy-induced bone loss as indicated by increased BV/TV and reduced Oc.N in C57BL/6 mice (27). However, a study by Liang *et al* (28) demonstrated different findings, where i.p. injection of CQ (50 mg/kg/day) for 1 month reduced BV/TV, Tb.N and trabecular thickness, and increased the trabecular pattern factor but caused no change in the structure model index of ovariectomised mice (28). Another animal study reported that the administration of CQ at 12.5 mg/kg for 60 days was insufficient to induce any changes in bone markers (ALP and TRAP) and bone microstructure [BV/TV, Tb.N, trabecular separation (Tb.Sp) and trabecular bone area] in ovariectomised rats (29).

Using the ovariectomised animal model, several studies have demonstrated that the presence of CQ might enhance or weaken the bone-protecting effects of other compounds. For example, CQ acted synergistically with curcumin (a compound isolated from turmeric root) in ameliorating bone loss by increasing ALP expression and decreasing TRAP expression, resulting in good bone microarchitecture in ovariectomised rats (29). On the contrary, the combination of CQ with quercetin (a polyphenol flavonoid found in several fruits, vegetables, leaves, seeds and grain) (26) and icariin (a natural flavonoid glycoside extracted from *Herba epimedii* with a bone-protecting property) (28) weakened their actions on attenuating bone loss in ovariectomised mice. The co-administration of CQ with quercetin (50 mg/kg; oral administration) reduced P1NP in ovariectomised rats compared with those treated with CQ only (26). When CQ was co-administered with icariin, CQ diminished the icariin-induced increase of bone mass in ovariectomised mice (28).

RANKL is a cytokine essential for osteoclast differentiation (30). A study looked at 8-week-old mice that were injected (i.p.) with RANKL (1 mg/kg) at 24 h intervals for 3 days to stimulate osteoclasts for bone resorption. The injection of CQ (50-200 mg/kg) was given 1 h before every RANKL injection. All bone microstructural parameters, including BV/TV, Tb.Th, Tb.N and Tb.Sp, were measured after the last injection of RANKL. The findings revealed marked increases in BV/TV and Tb.N but a reduction in Tb.Sp in RANKL-induced bone loss in mice (30).

D-galactose is a monosaccharide sugar that forms lactose when combined with glucose. It causes pathological changes that resemble natural ageing, including reduced bone mass (31). The prolonged administration of D-galactose increases advanced glycation end products, receptors for advanced glycation end products and nicotinamide adenine dinucleotide phosphate oxidase, leading to the excessive accumulation of reactive oxygen species (ROS) and mitochondrial damage (32). In D-galactose-induced osteoporotic rats, treatment with 10 mg/kg CQ for 4 consecutive weeks increased tibial BMD, femoral BMD, serum phosphate levels and OPG, but decreased serum calcium, ALP, OCN, RANKL, cathepsin K (CTSK), TRAP and the percentage of bone loss (33).

In a study using female mice with autosomal dominant osteopetrosis type II mutation (ADO2) that developed osteopetrosis, the administration of CQ (1-10 mg/kg/day) via drinking water for 6 months increased osteoclast activity, as evidenced by increased TRAP expression. However, no change was observed in the improvement of the osteoporotic bone phenotype of ADO2 mice (34). Foxo1 is a transcription factor abundantly found in bone, indicating its role as a mediator for skeletal development. Foxo1 is highly expressed in bone tissues, and its silencing impairs skeleton formation (35,36). The effects of CQ (2 mg/kg given every 2 days) via injection (i.p.) on bone have been investigated in Foxo1-overexpressing mice. Foxo1-overexpressing mice treated with CQ exhibited lower BV/TV and Tb.N but higher Tb.Sp in the femur as compared to the wild-type controls. The presence of CQ also downregulated the expression levels of several osteogenic markers, including Runt-related transcription factor 2 (Runx-2), osterix and ALP. The findings of the study reiterated that CQ reversed the favourable osteogenic effects of Foxo1 overexpression (36).

Several studies have demonstrated the effects of HCQ on bones. In a mouse model of arthritis, HCQ was administered by oral gavage at a dose of 80 mg/kg daily for 15 days. Oc.N,

Table II. Effects of CQ or HCQ on bone health in animals.

First author/s, year	Animal model(s)	Treatment (dose, route of administration and duration)	Findings	(Refs.)
Fischer and Fitch, 1975	Adult and 8-day-old male and female Sprague-Dawley rats 1-day-old leghorn chickens	[3-^{14}C]CQ (96.4 μg; single i.p. injection, 30 min)	Affinity of CQ and its metabolites for bone ↑	(21)
Aoki *et al*, 2020	Dexamethasone-induced mice	CQ (50 mg/kg/day; i.p.; 7 days)	TRAP activity ↓, ALP ↔	(23)
Lin *et al*, 2016	Dexamethasone-induced mice	CQ (2 mg/kg; every 2 days; i.p.; 8 weeks)	BV/TV ↑, Tb.N ↑, Oc.N ↓, Ob.N ↔, Ob.S ↔	(24)
	Ovariectomised mice		BV/TV ↑, Tb.N ↑, Oc.N ↓, Ob.N ↔, Ob.S ↔	
Xiong *et al*, 2023	Ovariectomised rats	CQ (10 mg/kg; i.p.; twice a week; 8 weeks)	P1NP ↔, CTX ↓, Beclin 1 ↓, LC3II/I ratio ↓, p62/SQSTM1 ↑, Oc.N ↓	(26)
		CQ (10 mg/kg; i.p.; twice a week; 8 weeks) + quercetin (50 mg/kg/day; oral; 8 weeks)	P1NP ↓, CTX ↓, Beclin 1 ↓, LC3II/I ratio ↓, p62/SQSTM1 ↑, Oc.N ↓	
Xiu *et al*, 2014	Ovariectomised mice	CQ (50 mg/kg/day; i.p.; 28 days)	Oc.N ↓, BV/TV ↑	(27)
Liang *et al*, 2019	Ovariectomised mice	CQ (50 mg/kg/day; i.p.; 1 month)	BV/TV ↓, Tb.N ↓, Tb.Th ↓, SMI ↔, Tb.Pf ↑, Oc.N ↑	(28)
		CQ (50 mg/kg/day; i.p.; 1 month) + icariin (50 mg/kg/day; i.p.; 1 month)	BV/TV ↔, Tb.N ↔, Tb.Th ↔, SMI ↔, Tb.Pf ↔, Oc.N ↔	
Ke *et al*, 2020	Ovariectomised rats	CQ (12.5 mg/kg/day; i.p.; 60 days)	BV/TV ↔, Tb.N ↔, Tb.Sp ↔, Tb.Ar ↔, TRAP ↔, ALP ↔	(29)
		CQ (12.5 mg/kg/day; i.p.; 60 days) + curcumin (110 mg/kg/day; oral; 60 days)	BV/TV ↑, Tb.N ↑, Tb.Sp ↓, Tb.Ar ↑, TRAP ↓, ALP ↑	
Al-Bari *et al*, 2012	RANKL-induced mice	CQ (50-200 mg/kg/day; 3 days)	BV/TV ↑, Tb.Th ↑, Tb.N ↑, Tb.Sp ↓	(30)
Mahmoud *et al*, 2021	D-galactose-induced osteoporotic rats	CQ (10 mg/kg/day; oral; 4 weeks)	BMD ↑, serum calcium ↓, serum phosphate ↑, ALP ↓, OCN ↓, OPG ↑, RANKL ↓, CTSK ↓, TRAP ↓, ERK ↓, percentage of bone loss ↓	(33)
Alam *et al*, 2021	Female ADO2 mice	CQ (1-10 mg/kg/day; oral; 6 months)	BMC ↔, BMD ↔, BV/TV ↔, Tb.Th ↔, Tb.N ↔, Tb.Sp ↔, CTX ↔, TRAP ↑, P1NP ↔	(34)
Jiang *et al*, 2023	Foxo1-overexpressing mice	CQ (2 mg/kg; every 2 days; i.p.)	BV/TV ↓, Tb.N ↓, Tb.Sp ↑, Runx-2 ↓, OSX ↓, ALP ↓	(36)
Li *et al*, 2023	Arthritic mice	HCQ (80 mg/kg/day; oral; 15 days)	Oc.N ↓, Oc.S ↓, CTX-1 ↓	(37)
Topak *et al*, 2023	Rats subjected to osteotomy at the right femur	HCQ sulphate (0.45-3 mg; oral; 5 days)	Radiological score ↓, ALP ↓, callus/diaphysis ratio ↓, callus development ↓, BMD ↓, CAT ↑, SOD ↑, GPx ↑, MDA ↑	(38)
Önaloğlu *et al*, 2024	Rats subjected to open diaphyseal femur fractures	HCQ sulphate (160 mg/kg/day; oral; 2 or 4 weeks)	MDA ↑, ALP ↓, OCN ↔, OPN ↔, CTSK ↑, TRAP ↑, histological healing scores ↔, radiological scores ↔, total callus diameter to femoral bone diameter ↓	(39)
Tekçe *et al*, 2024	Rats subjected to a closed fracture at the right femur	HCQ (3-10 mg/kg; two times per day; 15 days)	Histological scoring ↔	(40)

ADO2, autosomal dominant osteopetrosis type II mutation; ALP, alkaline phosphatase; BMC, bone mineral content; BMD, bone mineral density; BV/TV, bone volume/tissue volume; CAT, catalase; CQ, chloroquine; CTSK, cathepsin K; CTX-1, cross-linked C-telopeptide of type 1 collagen; GPx, glutathione peroxidase; HCQ, hydroxychloroquine; i.p., intraperitoneal; MDA, malondialdehyde; Ob.N, osteoblast number; Ob.S, osteoblast surface; OCN, osteocalcin; Oc.N, osteoclast number; Oc.S, osteoclast surface; OPG, osteoprotegerin; OPN, osteopontin; OSX, osterix; P1NP, propeptide of type I procollagen; p62/SQSTM1, sequestosome-1; RANKL, receptor activator of nuclear factor κ-B ligand; Runx-2, Runt-related transcription factor 2; SMI, structure model index; SOD, superoxide dismutase; Tb.Ar, trabecular bone area; Tb.N, trabecular number; Tb.Pf, trabecular pattern factor; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; TRAP, tartrate-resistant acid phosphatase; ↑, increase/upregulate; ↓, decrease/downregulate; ↔, no change.

osteoclast surface (Oc.S) and CTX in the arthritic mice treated with HCQ were decreased compared with those in mice treated with normal saline (37). However, HCQ has been reported to induce oxidative stress, thereby impairing fracture healing (38). A study by Topak *et al* (38) utilised male Wistar albino rats with osteotomy at the mid-distal region of the right femur, which were administered HCQ sulphate orally at various doses (0.45-3 mg) for 5 days, and revealed that this resulted in a decrease in the radiological score, ALP, callus/diaphysis ratio, callus development and BMD. Mechanistically, the circulating antioxidant enzyme activities [catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx)] were increased, which could be a response to neutralise the overwhelming oxidative stress (as lipid peroxidation was elevated) in rats treated with HCQ sulphate (38). In male rats subjected to open diaphyseal femur fracture, the oral administration of HCQ sulphate (160 mg/kg) increased malondialdehyde levels. In HCQ-treated animals, no improvement was observed in the histological healing scores, whereby the cartilage callus tissue disappeared and the area of immature bone tissue was increased. The total callus diameter at the femur was lower in the HCQ group compared with the control group. Levels of ALP were decreased, whereas OCN and osteopontin (OPN) levels were unchanged. Meanwhile, CTSK and TRAP were increased in the HCQ group relative to the control group (39). Another recent study utilised male rats with a closed fracture created at the proximal region of the femur. Treatment with HCQ (3-10 mg/kg) two times per day (once in the morning and once in the evening) for 15 days did not improve the histologic scoring compared with the negative control (40).

In conclusion, bone is an important storage organ for CQ. The aforementioned studies revealed that different doses, treatment frequency and duration affect the skeletal actions of CQ. Long-term daily administration adversely affects bone health. CQ has more prominent effects on osteoclastic activity and bone resorption compared with osteoblastic activity and bone formation. In addition, CQ may serve as a modulator to reduce bone resorption in osteoporotic and arthritic conditions whilst decreasing osteoblastic activity and increasing osteoclastic activity during osteopetrosis. Although CQ may appear to be a potential strategy in the treatment of osteoporosis and osteopetrosis, CQ showed no beneficial effect in inducing fracture healing.

5. *In vitro* studies: Effects of CQ and HCQ on bone cells

Osteoblasts. *In vitro* studies reporting the effects of CQ and HCQ on bone-related parameters using osteoblasts are summarised in Table III. Incubation of CQ at a concentration of 5 μM for 48 h did not exhibit a cytotoxic effect on murine pre-osteoblast MC3T3-E1 cells (41). The presence of CQ (5-50 μM) has been demonstrated to inhibit osteogenic differentiation (confirmed by reduced ALP activity, and reduced OCN and Runx-2 expression) in MC3T3-E1 cells, human dental pulp mesenchymal stem cells and bone marrow mesenchymal stem cells (BMSCs) (28,42-45). CQ (5-10 μM) suppressed mineralisation in BMSCs isolated from normal mice (28,45). Similarly, BMSCs extracted from CQ-injected mice exhibited less mineralised nodules and osteogenic differentiation ability, as indicated by reduced expression of bone morphogenetic protein-2 and Runx-2 (28). However, CQ (50 μM) augmented the formation of mineralised nodules at the late stage of bone formation (43).

The presence of CQ weakened the efficiency of icariin, lactoferrin (a glycoprotein derived from milk), triiodothyronine (a thyroid hormone) and bone morphogenetic protein-9 (BMP9) in attenuating bone loss. BMSCs treated with CQ and icariin exhibited reduced ALP expression and mineralised nodule formation compared with those treated with icariin alone. Likewise, BMSCs isolated from mice co-treated with CQ and icariin exhibited reduced osteogenic differentiation and mineralisation compared with BMSCs isolated from mice administered icariin alone (28). In another study, lactoferrin was found to increase the expression levels of P1NP (a bone formation marker) in MC3T3-E1 cells, which was reduced following combined therapy with CQ and lactoferrin (46). Yi *et al* (47) revealed that CQ reversed triiodothyronine-promoted cell viability and ALP activity, as well as proliferating cell nuclear antigen, type I collagen and OCN expression in cranial osteoblasts. CQ also effectively inhibited ALP activity and matrix mineralisation promoted by BMP9 in BMSCs (48). Nonetheless, HCQ further enhanced osteogenesis in trehalose-treated co-culture of MC3T3-E1 cells and bone marrow monocytes/macrophages (BMMs) as the expression levels of Runx-2, activating transcription factor 4 and OPN were increased (49).

TNF-α is an important inflammatory cytokine that inhibits bone formation, enhances bone resorption and attenuates osteoblast survival (50). Cadmium is a toxic heavy metal known to be an environmental pollutant that can induce apoptosis in osteoblasts (51). Incubation with CQ reduced the viability of MC3T3-E1 cells induced by TNF-α (50) and rat cranial osteoblasts induced by cadmium (51). The effects of HCQ on osteoblasts have been evaluated in a study by Both *et al* (52). HCQ at a concentration of 5 μg/ml inhibited the differentiation of human mesenchymal stromal cells into osteoblasts at day 7 and inhibited osteoblast mineralisation at day 18. Human mesenchymal stromal cells exhibited less cell surface attachment, but no cytoskeletal malformations were observed after HCQ treatment (52).

Therefore, CQ alone did not exert cytotoxic effects on osteoblasts. However, *in vitro* studies consistently demonstrated that CQ and HCQ inhibited osteogenic differentiation and mineralisation, with the exception that a higher dose of CQ (50 μM) increased mineralisation. The presence of CQ and HCQ might strengthen or weaken the bone sparing effects of other compounds (28,46-49).

Osteoclasts. Murine monocyte/macrophage cells (RAW264.7), BMMs or osteoclast precursors can differentiate into osteoclasts under the influence of macrophage colony-stimulating factor (M-CSF), RANKL or inflammatory cytokines (30). *In vitro* studies have consistently shown the inhibitory effects of CQ on osteoclast differentiation and function (Table IV). Ke *et al* (29) reported that CQ (2 μM) treatment caused no reduction in Oc.N, TRAP, MMP-9 and CTSK in BMMs incubated with M-CSF and RANKL (29). Al-Bari *et al* (30) reported that CQ (1-10 μM) treatment inhibited the formation of TRAP-positive multinucleated osteoclasts and resorption pits in a dose-dependent manner using BMMs stimulated by

Table III. Studies investigating the effects of CQ and HCQ in osteoblasts.

First author/s, year (Refs.)	Type of cells	Treatment	Findings	
Xu *et al*, 2021	MC3T3-E1 cells	CQ (5 μM)	Cell viability ↔, LC3II/I ratio ↔, apoptosis ↔, caspase-3 ↔, PARP ↔, ROS ↔, Nox4 ↔	(41)
Zhang *et al*, 2017	MC3T3-E1 cells	CQ (10 μM)	ALP ↓, mineralisation ↓, LC3II ↓, apoptosis ↑	(45)
Zhang *et al*, 2023	MC3T3-E1 cells	CQ (10 μM)	LC3II ↑, apoptosis ↑	(76)
Li *et al*, 2024	MC3T3-E1 cells	CQ (20 μM)	ALP ↓, LC3II/I ratio ↑, p62/SQSTM1 ↑, apoptosis ↑, Bax ↑, Bcl-2 ↓, caspase-3 ↑, p53 ↑, T-AOC ↓, SOD ↓, NO ↑	(42)
Qiu *et al*, 2023	MC3T3-E1 cells	CQ (50 μM)	ALP ↓, mineralisation ↑, autophagosome ↓, autolysosome ↑, p62/SQSTM1 ↑, Lamp1 ↑, Bcl-2 ↔	(43)
Pantovic *et al*, 2013	Human dental pulp mesenchymal stem cells	CQ (20 μM)	ALP ↓, OCN ↓, Runx-2 ↓	(44)
Liang *et al*, 2019	BMSCs isolated from BALB/c mice	CQ (5 μM)	ALP ↓, mineralisation ↓	(28)
		CQ (5 μM) + icariin (10 μM)	ALP ↓, mineralisation ↓	
	BMSCs isolated from BALB/c mice treated with CQ	N/A	Mineralisation ↓, Runx-2 ↓, BMP-2 ↓, Beclin 1 ↔, LC3II/I ratio ↓, p62/SQSTM1 ↑	
	BMSCs isolated from BALB/c mice treated with CQ and icariin	N/A	Mineralisation ↓, Runx-2 ↓, BMP-2 ↓, Beclin 1 ↓, LC3II/I ratio ↓, p62/SQSTM1 ↑	
Zhang *et al*, 2020	MC3T3-E1 cells	CQ (50 mM) + lactoferrin (100 μg/ml)	P1NP ↓, autophagy ↓, p-p38 MAPK ↓, Nbr1 ↑	(46)
Yi *et al*, 2020	Cranial osteoblasts	CQ (5 μM) + triiodothyronine (100 nM)	Cell viability ↓, ALP activity ↓, PCNA ↓, COL1 ↓, OCN ↓	(47)
Zhao *et al*, 2021	BMP-9-induced BMSCs	CQ (10 μM)	ALP ↓, mineralisation ↓	(48)
Ni *et al*, 2020	TNF-α-induced MC3T3-E1 cells	CQ (10 μM)	Cell viability ↓, LC3II/I ratio ↓, caspase-3 ↑	(50)
Ni *et al*, 2020	TNF-α-induced MC3T3-E1 cells	CQ (10 μM)	LC3II ↑, p62/SQSTM1 ↑, cleaved PARP ↑	(77)
Liu *et al*, 2016	Cadmium-induced rat cranial osteoblasts	CQ (5 μM)	Cell viability ↓, LC3II ↓, apoptosis ↑	(51)
Both *et al*, 2018	Human mesenchymal stromal cells	HCQ (5 μg/ml)	ALP activity ↓, alizarin red and von Kossa staining ↓, cell-surface attachment ↓, cytoskeletal malformations (actin) ↔, apoptosis ↔	(52)
Xu *et al*, 2020	Co-culture of MC3T3-E1 cells and BMMs	HCQ (dose unspecified) + trehalose (50 mM)	Runx-2 ↑, OPN ↑, ATF4 ↑, Beclin 1 ↓, LC3II/I ↑	(49)
Yang *et al*, 2023	BMSCs isolated from C57BL/6 mice	HCQ (dose unspecified)	Autolysosome ↓, disruption of mitochondrial membrane potential ↑	(79)

Table III. Continued.

First author/s, year	Type of cells	Treatment	Findings	(Refs.)
Liu *et al*, 2021	BMSCs isolated from BALB/c mice	HCQ (10 μM)	LC3II ↑, p62/SQSTM1 ↑, cell senescence ↑	(78)

ALP, alkaline phosphatase; ATF4, activating transcription factor 4; BMMs, bone marrow monocytes/macrophages; BMP-2, bone morphogenetic protein-2; BMP-9, bone morphogenetic protein-9; BMSCs, bone marrow mesenchymal stem cells; COL1, type I collagen; CQ, chloroquine; HCQ, hydroxychloroquine; Lamp1, lysosome-associated membrane glycoprotein 1; N/A, not application; Nbr1, neighbour of Brca1 gene; NO, nitric oxide; Nox4, nicotinamide adenine dinucleotide phosphate oxidase 4; OCN, osteocalcin; OPN, osteopontin; PARP, poly (adenosine diphosphate-ribose) polymerase; PCNA, proliferating cell nuclear antigen; p-, phosphorylated; P1NP, propeptide of type I procollagen; p62/SQSTM1, sequestosome-1; ROS, reactive oxygen species; Runx-2, Runt-related transcription factor 2; SOD, superoxide dismutase; T-AOC, total antioxidant capacity; ↑, increase/upregulate; ↓, decrease/downregulate; ↔, no change.

M-CSF and RANKL. The expression of nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), the master transcription factor of osteoclast differentiation, was also inhibited by CQ treatment (30). Two studies have shown that CQ (10 μM) reduced Oc.N and the resorption pit area in BMMs induced by M-CSF and lipopolysaccharides (LPS) or RANKL (27,53). Furthermore, the expression levels of MMP-9, TRAP and CTSK were decreased in the presence of CQ (53). When using RAW264.7 cells stimulated by RANKL, CQ (10 μM) treatment attenuated the formation of osteoclasts, actin ring and resorption pits. In addition, CQ treatment inhibited the RANKL-induced upregulation of osteoclast differentiation factors, including MMP-9, TRAP, CTSK and dendritic cell-specific transmembrane protein (54,55). Likewise, CQ arrested M-CSF- and RANKL-induced osteoclast formation from the precursor cells (56).

Similar to the findings observed in animal studies, CQ might enhance or weaken the inhibition of osteoclastogenesis when given as combined therapy with other agents *in vitro*. The combination of CQ and curcumin conferred synergistic effects in reducing osteoclast differentiation in BMM stimulated by M-CSF and RANKL (29). BMMs stimulated by M-CSF and RANKL were treated with either 1 or 10 μmol/l CQ, and reduced osteoclast formation, lacunar resorption area and TRAP expression levels were observed (57,58). The co-treatment with a lower dose of CQ (1 μmol/l) and OPG (40 ng/ml) reduced the ability of OPG to reduce the lacunar resorption area, CTSK and TRAP expression levels (58). However, co-treatment with a higher dose of CQ (10 μmol/l) and OPG (40 ng/ml) improved the ability of OPG to inhibit osteoclastogenesis, as indicated by the further reduction of TRAP-positive cells and lacunar resorption area (57).

Dexamethasone increases osteoclast formation by stimulating the proliferation and differentiation of osteoclast precursors into mature osteoclasts (23,24). The TRAP activity, osteoclast formation and bone resorption area were decreased in dexamethasone-induced osteoclasts after incubation with CQ (5-10 μM) (23,24). IL-17A is a pro-inflammatory cytokine produced by Th17 cells, which induces osteoclastogenesis and bone resorption (59). The increased mature osteoclast formation of RAW264.7 cells induced by IL-17A was suppressed by 30 μM CQ (60). Osteoclastoma, also known as a giant cell tumour of the bone, is a rare and benign (non-cancerous) tumour developed predominantly at the ends of the femur or the upper end of the tibia leading to local bone destruction (61). The presence of CQ (25 μmol/l) increased lysosomal pH and prevented the proteolytic processing of CTSK in human osteoclasts extracted from fresh osteoclastoma tissue (61). In addition, the shedding of titanium particles from wear implants into hard or soft tissues may stimulate the inflammatory response and osteoclast differentiation (62). In macrophage (KG-1a) cells, the upregulation of RANKL protein induced by a pure titanium particle was inhibited by the addition of 100 μM CQ (62).

Another study utilised two different cell culture models to resemble osteoporotic conditions. Heterozygous mice with a point mutation (R740S) in the a3 subunit of V-ATPase (+/R740S) displayed mild osteopetrosis and impaired bone resorption. The TRAP-positive cells were reduced in BMMs isolated from mice carrying the R740S mutation stimulated

Table IV. Effects of CQ or HCQ on osteoclasts.

First author/s, year	Types of cells	Treatment	Findings	(Refs.)
Al-Bari *et al*, 2012	BMMs stimulated by M-CSF and RANKL	CQ (1-10 μM)	TRAP-positive cells ↓, resorption pit area ↓, NFATc1 ↓, TUNEL-positive cells ↑ (at high dose)	(30)
Hu *et al*, 2022	BMMs stimulated by M-CSF and LPS	CQ (10 μM)	Oc.N ↓, MMP-9 ↓, TRAP ↓, CTSK ↓, LC3II/I ratio ↑, p62/SQSTM1 ↑, TRAF3 ↑	(53)
Xiu *et al*, 2014	BMMs stimulated by M-CSF and RANKL	CQ (10 μM)	Oc.N ↓, resorption pit area ↓, TRAF3 ↑	(27)
Hu *et al*, 2016	BMMs stimulated by M-CSF and RANKL	CQ (10 μM)	LC3II/I ratio ↑, mTOR ↑, p-mTOR ↑	(80)
Sun *et al*, 2021	RAW264.7 cells stimulated by RANKL	CQ (10 μM)	TRAP-positive cells ↓, resorption pit ↓, MMP-9 ↓, TRAP ↓, CTSK ↓, LC3II/I ratio ↑	(54)
Wu *et al*, 2020	RAW264.7 cells stimulated by RANKL	CQ (10 μM)	TRAP-positive cells ↓, actin ring formation ↓, NFATc1 ↔, c-Fos ↔, DC-STAMP ↓	(55)
Ito *et al*, 2005	RAW264.7 cells stimulated by RANKL	CQ (150 μM)	c-Fos ↔	(68)
Yao *et al*, 2017	Osteoclast precursors stimulated by M-CSF and RANKL	CQ (dose unspecified)	Oc.N ↓, TRAF3 ↑	(56)
Okusha *et al*, 2020	Murine monocytic (RAW-D) cells stimulated by RANKL	CQ (10 μM)	c-fms ↑, RANK ↑	(69)
Tran *et al*, 2020	Murine monocytic (RAW-D) cells stimulated by RANKL	CQ (1-30 μM)	c-fms ↑, RANK ↑	(70)
Ke *et al*, 2020	BMMs stimulated by M-CSF and RANKL	CQ (2 μM)	Cell proliferation ↔, Oc.N ↔, TRAP ↔, MMP-9 ↔, CTSK ↔	(29)
		CQ (2 μM) + curcumin (5-15 μM)	Cell proliferation ↓, Oc.N ↓, TRAP ↓, MMP-9 ↓, CTSK ↓	
Zhao *et al*, 2020	BMMs stimulated by M-CSF and RANKL	CQ (1 μmol/l)	TRAP-positive cells ↓, lacunar resorption area ↓, CTSK ↔, TRAP ↓, LC3II ↑, p62/SQSTM1 ↔, autophagosome ↑	(58)
		CQ (1 μmol/l) + OPG (40 ng/ml)	TRAP-positive cells ↔, lacunar resorption area ↑, CTSK ↑, TRAP ↑, LC3II ↑, p62/SQSTM1 ↑, autophagosome ↑	
Tong *et al*, 2019	BMMs stimulated by M-CSF and RANKL	CQ (10 μmol/l)	TRAP-positive cells ↓, lacunar resorption area ↓, autophagosome ↑, Atg5 ↔, Atg7 ↔, Atg12 ↔, Atg13 ↔	(57)
		CQ (10 μmol/l) + OPG (40 ng/ml)	TRAP-positive cells ↓, lacunar resorption area ↓, autophagosome ↓, Atg12 ↓, Beclin 1 ↑, p62/SQSTM1 ↑, LC3II ↓, p-mTOR ↓, Raptor ↓, GβL ↑, p-AMPK ↑, TSC2 ↓, Rheb ↓, p-p70S6K ↓	
Aoki *et al*, 2020	Dexamethasone-induced BMMs stimulated by M-CSF and RANKL	CQ (5 μM)	TRAP activity ↓	(23)
Lin *et al*, 2016	Dexamethasone-induced BMMs stimulated by M-CSF and RANKL	CQ (10 μM)	TRAP-positive cells ↓, bone resorption area ↓	(24)
Ke *et al*, 2018	IL-17A-induced RAW264.7 cells stimulated by M-CSF and RANKL	CQ (30 μM)	Oc.N ↓	(60)
Rieman *et al*, 2001	Human osteoclasts isolated from fresh osteoclastoma tissue	CQ (25 μmol/l)	Lysosomal pH ↑, proteolytic processing of CTSK ↓	(61)
Su *et al*, 2018	Titanium wear particle-induced KG-1a cells	CQ (100 μM)	RANKL ↓	(62)

Table IV. Continued.

First author/s, year	Types of cells	Treatment	Findings	(Refs.)
Voronov *et al*, 2013	BMMs isolated from mice carrying the R740S mutation stimulated by M-CSF and RANKL	CQ (10 μM)	TRAP-positive cells ↓, RCAN1 ↑	(63)
Alam *et al*, 2021	BMMs isolated from $ADO2^{+/+}$ mice stimulated by M-CSF and RANKL	CQ (30 nM)	Osteoclast formation ↔, osteoclast survival ↓, CTX ↓, resorption pit area ↔	(34)
	BMMs isolated from $ADO2^{+/-}$ mice stimulated by M-CSF and RANKL	CQ (30 nM)	Osteoclast formation ↓, osteoclast survival ↔, CTX ↑, resorption pit area ↑	
	BMMs isolated from $ADO2^{+/+}$ mice stimulated by M-CSF and RANKL	DCQ (100 nM)	Osteoclast formation ↓, osteoclast survival ↓, CTX ↑, resorption pit area ↑	
	BMMs isolated from $ADO2^{+/-}$ mice stimulated by M-CSF and RANKL	DCQ (100 nM)	Osteoclast formation ↓, osteoclast survival ↔, CTX ↑, resorption pit area ↑	
Both *et al*, 2018	PBMC-sorted monocytes	HCQ (5 μg/ml)	Multinuclear osteoclasts ↓, surface resorption ↓, CTSK ↓, TM7SF4 ↑, lysosomal membrane permeabilization ↑, DAPI staining ↔, Bax/Bcl-2 ↔, caspase 3 ↔	(16)
Huang *et al*, 2023	PBMCs isolated from patients with rheumatoid arthritis stimulated by M-CSF and RANKL	HCQ (dose unspecified)	Cell viability ↓, CTR ↓, TRAP ↓, CTSK ↓, MMP-9 ↓, apoptosis ↑, CCL20 ↓, CXCL8 ↓, HIF-1α ↔, IL-1 ↓, IL-6 ↓, IL-17 ↓, TNF-α ↓, mTOR ↔, LC3I/II ratio ↔, Atg5 ↔, Beclin 1 ↔	(64)
Xu *et al*, 2020	Co-culture of MC3T3-E1 cells and BMMs	HCQ (dose unspecified) + trehalose (50 mM)	Oc.N ↓, OPG ↑, OPG/RANKL ↑	(49)
Lee *et al*, 2004	Co-culture of fibroblast-like synoviocytes and PBMCs isolated from healthy volunteers	HCQ (1-20 μM)	Cell proliferation ↔, Oc.N ↔	(65)

ADO2, autosomal dominant osteopetrosis type II mutation; AMPK, 5'AMP-activated protein kinase; Atg, autophagy-related proteins; BMMs, bone marrow monocytes/macrophages; CCL20, chemokine (C-C motif) ligand 20; c-fms, macrophage colony-stimulating factor receptor; c-Fos, Fos proto-oncogene; CQ, chloroquine; CTR, calcitonin receptor; CTSK, cathepsin K; CTX, cross-linked C-telopeptide of type 1 collagen; CXCL8, CXC motif chemokine ligand 8; DCQ, desethylchloroquine; DC-STAMP, dendritic cell-specific transmembrane protein; GβL, G protein β-subunit-like protein; HCQ, hydroxychloroquine; HIF-1α, hypoxia-inducible factor 1α; LPS, lipopolysaccharides; M-CSF, macrophage colony-stimulating factor; NFATc1, nuclear factor of activated T-cells cytoplasmic 1; Oc.N, osteoclast number; OPG, osteoprotegerin; p-, phosphorylated; PBMCs, peripheral blood mononuclear cells; p-mTOR, phosphorylated mammalian target of rapamycin; p-p70S6K, phosphorylated p70 ribosomal protein S6 kinase; p62/SQSTM1, sequestosome-1; RANK, receptor activator of nuclear factor κ-B; RANKL, receptor activator of nuclear factor κ-B ligand; RCAN1, regulator of calcineurin; Rheb, Ras homolog enriched in brain; TM7SF4, transmembrane 7 superfamily member 4; TRAF3, tumour necrosis factor receptor-associated factor 3; TRAP, tartrate-resistant acid phosphatase; TSC2, tuberous sclerosis complex 2; ↑, increase/upregulate; ↓, decrease/downregulate; ↔, no change.

by M-CSF and RANKL and exposed to CQ (10 μM). The mechanism underlying the anti-osteoclastogenic action of CQ was modulated by the increased level of the regulator of calcineurin, followed by NFATc1 inhibition (63). Alam *et al* (34) isolated BMMs from the femurs and tibias of $ADO2^{+/+}$ and $ADO2^{+/-}$ mice, displaying normal and impaired osteoclastic bone resorption, respectively. Exposure to CQ (30 nM) caused reductions in CTX expression levels and osteoclast survival in normal mice, as well as a reduction in osteoclast formation and an increase in CTX and resorption pit formation in $ADO2^{+/-}$ mouse-derived cells. Treatment with desethylchloroquine (DCQ; a CQ metabolite) at 100 nM resulted in decreased osteoclast formation and survival but increased CTX expression levels and resorption pit formation in BMMs extracted from normal mice. Similar concentrations of DCQ decreased osteoclast formation, and increased CTX expression levels and resorption pit formation, without affecting osteoclast survival in $ADO2^{+/-}$ mouse-derived cells (34). The findings of these studies indicate that osteoclast activity was enhanced by CQ and DCQ under osteoporotic conditions but with fewer numbers of osteoclasts. Such observations might be due to CQ and DCQ reducing the less efficient osteoclast, leaving a subpopulation of highly active osteoclasts.

Four studies have examined the effects of HCQ on osteoclast formation and activity. Using human peripheral blood mononuclear cell (PBMC)-sorted monocytes to differentiate into osteoclasts, the presence of HCQ (5 μg/ml) was revealed to inhibit the formation of multinuclear osteoclasts, surface resorption and expression of CTSK while increase transmembrane 7 superfamily member 4 (TM7SF4, an osteoclast fusion marker) expression (16). In another study using PBMCs from patients with rheumatoid arthritis, HCQ reduced cell viability and osteoclastogenic gene expression (calcitonin receptor; TRAP; CTSK and MMP-9) when culturing in a medium containing M-CSF and RANKL (64). Xu *et al* (49) further demonstrated that HCQ reduced osteoclast formation in a co-culture system containing MC3T3-E1 cells and BMMs (49). However, one study reported contradictory outcomes, whereby HCQ (1-20 μM) did not inhibit cell proliferation and osteoclast formation in the co-culture of fibroblast-like synoviocytes and PBMCs in the presence of M-CSF (65).

The effects of CQ and HCQ on osteoclasts have been established. CQ and HCQ exhibit positive effects on bone health by modulating osteoclast function and activity. They inhibit osteoclast proliferation and differentiation in an osteoclastogenic environment with or without an inducer but promote osteoclastogenesis during impaired bone resorption.

Osteocytes. To the best of our knowledge, only one study has evaluated the effects of CQ on osteocytes (Table V). Pre-incubation with 50 μM CQ did not exert a cytotoxic effect on osteocytic MLO-Y4 cells; however, CQ exacerbated hydrogen peroxide-induced cell death (66).

6. Mechanism of action of CQ and HCQ

M-CSF and receptor activator of nuclear factor κ-B (RANK)/RANKL/OPG pathways. The M-CSF and RANK/RANKL/OPG pathways are the most implicated mechanisms for monocyte/macrophage-to-osteoclast differentiation regulated by osteoblasts. M-CSF is a cytokine constitutively secreted by osteoblasts, which causes haematopoietic stem cells to differentiate into osteoclastic lineage in the presence of RANKL. RANK is a homotrimeric transmembrane receptor expressed on osteoclast precursors and mature osteoclasts. RANKL, produced by osteoblasts, binds to RANK as its receptor, eventually promoting osteoclast proliferation and maturation. Upon the interaction between RANKL and RANK, tumour necrosis factor receptor-associated factor 6 is recruited, stimulating a series of downstream targets, including NF-κB, NFATc1, MAPK and PI3K, which are responsible for osteoclast formation, activation and survival (67). The decoy receptor for RANKL, OPG, is also secreted by osteoblasts to prevent RANK/RANKL ligation and its subsequent reactions (67).

In D-galactose-induced bone loss, oral treatment with CQ (10 mg/kg) daily for 4 weeks replenished serum OPG levels and blunted the elevation of RANKL. Mechanistically, the elevated ERK expression in osteoporotic rats was mitigated by CQ administration (33). In RAW264.7 cells stimulated by RANKL, incubation with CQ (10-150 μM) did not cause any change in the expression of Fos proto-oncogene (c-Fos) (55,68). Lysosomes contribute to the proteolytic degradation of M-CSF receptor and RANK proteins, whereas the blocking of lysosomes by CQ enhances their expression in osteoclasts (69,70). In combination with other agent, HCQ co-treatment with trehalose further increased the expression levels of OPG and the OPG/RANKL ratio compared to trehalose alone in a co-culture of MC3T3-E1 cells and BMMs (49) (Fig. 2).

Autophagy machinery. Autophagy, also known as auto-phagocytosis, is a natural conserved process involved in the degradation and correction of intracellular proteins for the eradication of damaged or senescent macromolecules and organelles (71). Autophagy has two main regulators, namely 5'AMP-activated protein kinase (AMPK) as an activator and mTOR as an inhibitor blocking the auto-phagosomal membrane formation (72). Initiation/nucleation, elongation, maturation and degradation are the four major stages in autophagy regulated by autophagy-related proteins (Atg). Autophagy is initiated following an autophagic stimulus such as oxidative stress, hypoxia and lack of nutrients. The phagophore is formed by the combination of two multiprotein complexes: The class III PI3K nucleation complex [comprising Beclin 1, vacuolar protein sorting (Vps)34, Vps15 and Atg14] and the Unc-51-like kinases 1 (ULK1) initiation complex (comprising ULK1, Atg13 and Atg101). The elongation step involves the elongation of the phagophore enclosing cytoplasmic components that are non-functional, forming an autophagosome. The elongation and closure of autophagosomes are assisted by LC3 and Atg. LC3 exists as LC3I under physiological conditions and is converted to LC3II after conjugation with phospholipid phosphatidylethanolamine when autophagy is activated, thereby gradually increasing the LC3II/I ratio. Cytoplasmic components selected for degradation are tagged by selective autophagy co-receptors, such as sequestosome-1 (p62/SQSTM1) and neighbour of Brca1 gene (Nbr1)]. These co-receptors are recognised by LC3II, which directed them to the interior of the autophagosome. The maturation step involves the fusion of autophagosomes with lysosomes,

Table V. Effects of CQ in osteocytes.

First author/s, year	Types of cells	Treatment	Findings	(Refs.)
Kar *et al*, 2019	MLO-Y4 cells H_2O_2-induced MLO-Y4 cells	CQ (50 μM)	No cytotoxicity Cell death ↑	(66)

CQ, chloroquine; H_2O_2, hydrogen peroxide; ↑, increase/upregulate.

creating an acidic environment that facilitates the degradation of autophagosomal contents. In the final step, autophagosome contents are degraded by hydrolases from lysosomes, and the resulting molecules (lipids, amino acids and nucleotides) are reused to recycle cell components and produce energy (72,73).

Autophagy serves an important role in the homeostasis of bone cells (including osteoblasts, osteoclasts and osteocytes). Therefore, the dysfunction of autophagy is associated with the progression of bone loss and subsequent osteoporosis (74,75). The well-known autophagic inhibitor CQ blocks the fusion of autophagosome with lysosome and disrupts the acidic environment by inactivating acid hydrolases in lysosomes, favouring reduced osteoblast activity (74). Pretreatment with CQ (5 μM) was insufficient to cause any change in the LC3II/I ratio of MC3T3-E1 cells compared with control cells (41). Two studies have reported distinct outcomes; in one study, CQ (10 μM) reduced LC3II expression levels, while another study revealed increased LC3II expression in MC3T3-E1 cells, which may be due to different experimental conditions (acidic environment and high glucose) (45,76). Considering that only LC3II expression was assessed as an autophagy marker, determining the effects of CQ on autophagy under these conditions may be challenging. High concentrations of CQ (20-50 μM) increased LC3II expression and the accumulation of p62/SQSTM1, but decreased the formation of autophagosomes (42,43). Larger autolysosomes were accumulated and the expression levels of lysosome-associated membrane glycoprotein 1 (a lysosomal membrane component) were increased in MC3T3-E1 cells treated with CQ, indicating the impairment of auto-lysosomal degradation function (43). BMSCs obtained from CQ-injected mice exhibited unchanged Beclin 1 expression levels, a lower LC3II/I ratio and increased p62/SQSTM1 (28). The *in vitro* findings are consistent with those observed in an *in vivo* experimental setting. An animal study by Xiong *et al* (26) demonstrated that the expression levels of Beclin 1 and the LC3II/LC3I ratio were upregulated, and the levels of p62/SQSTM1 remained unchanged, indicating the activation of autophagy in ovariectomised rats compared with normal controls. The administration of CQ (10 mg/kg; i.p.) inhibited autophagy, thereby reducing Beclin 1 expression and the LC3II/I ratio but increasing the accumulation of p62/SQSTM1 in ovariectomised rats (26).

In the presence of other osteogenic agents, CQ suppresses autophagic and osteoblastic activities, resulting in the weakening of the bone-protecting effects of icariin (28), lactoferrin (46) and quercetin (26). BMSCs extracted from mice injected with CQ and icariin exhibited decreased Beclin 1 expression and LC3II/I ratios but increased p62/SQSTM1 expression (28). In a study by Zhang *et al* (46), the presence of CQ reduced lactoferrin-induced osteoblastic differentiation by suppressing autophagic activity via increased expression of Nbr1 and reduced activation of p38 MAPK (46). In ovariectomised rats injected with CQ and fed quercetin, the expression of Beclin 1 was further reduced compared with that in the animals treated with quercetin only (26). In TNF-α-stimulated MC3T3-E1 cells, the same researchers reported heterogenous outcomes on LC3II accumulation (decreased or increased) after treatment with CQ (10 μM) in two different studies (50,77). The discrepancy arises because of the different mechanisms of action involved. Connective tissue growth factor enhanced autophagy via protein kinase B and ERK activations; hence, LC3II did not accumulate when CQ was added (50). On the contrary, the inhibition of JAK2 suppressed autophagy, which was further inhibited upon CQ treatment, leading to LC3II accumulation (77). p62/SQSTM1 was upregulated in CQ-treated TNF-α-stimulated MC3T3-E1 cells, indicating the suppression of autophagy (77). In cadmium-stimulated MC3T3-E1 cells, CQ (5 μM) reduced the expression levels of LC3II and decreased cell viability (51). Treatment with HCQ also caused accumulation of LC3II and p62/SQSTM1, disrupted the mitochondrial membrane potential and blocked the formation of autolysosomes in BMSCs harvested from C57BL/6 mice, hindering the autophagic vacuole degradation of compromised mitochondria, which led to cellular senescence (78,79). HCQ treatment attenuated trehalose-induced autophagy, as shown by reduced Beclin 1 and increased LC3II in MC3T3-E1 cells (49). The aforementioned studies reiterated that CQ and HCQ suppressed autophagy, thereby reducing osteogenic differentiation. Co-treatment with CQ or HCQ further suppressed the autophagic activity and weakened the effects of other bone-protecting compounds. In addition, the inhibition of autophagy by CQ exacerbated the inhibition of osteogenic differentiation by an inflammatory mediator (TNF-α) and toxic heavy metal (cadmium).

Using BMMs or RAW264.7 cells induced by M-CSF and/or RANKL, the cell culture treated with CQ (1-10 μM) demonstrated inhibition of autophagy as indicated by the increased LC3II/I ratio, reduced transformation from autophagosomes to autolysosomes, as well as unchanged p62/SQSTM1 accumulation and Atg protein expression (54,57,58,80). The reduction of RANKL-induced osteoclast formation and function was mediated by the increase of tumour necrosis factor receptor-associated factor 3 (TRAF3; an anti-osteoclastic factor) (27,56) and mTOR protein levels (80). Furthermore, the LPS-inhibited TRAF3 protein level was recovered by autophagic inhibition by CQ, and a higher LC3II/I ratio and p62/SQSTM1 expression were noted (53). BMMs co-treated with CQ (1 μM) and OPG (40 ng/ml) inhibited autophagy as indicated by the high LC3II/I ratio, p62/SQSTM1 expression and autophagosomes (58). The combination of a higher dose

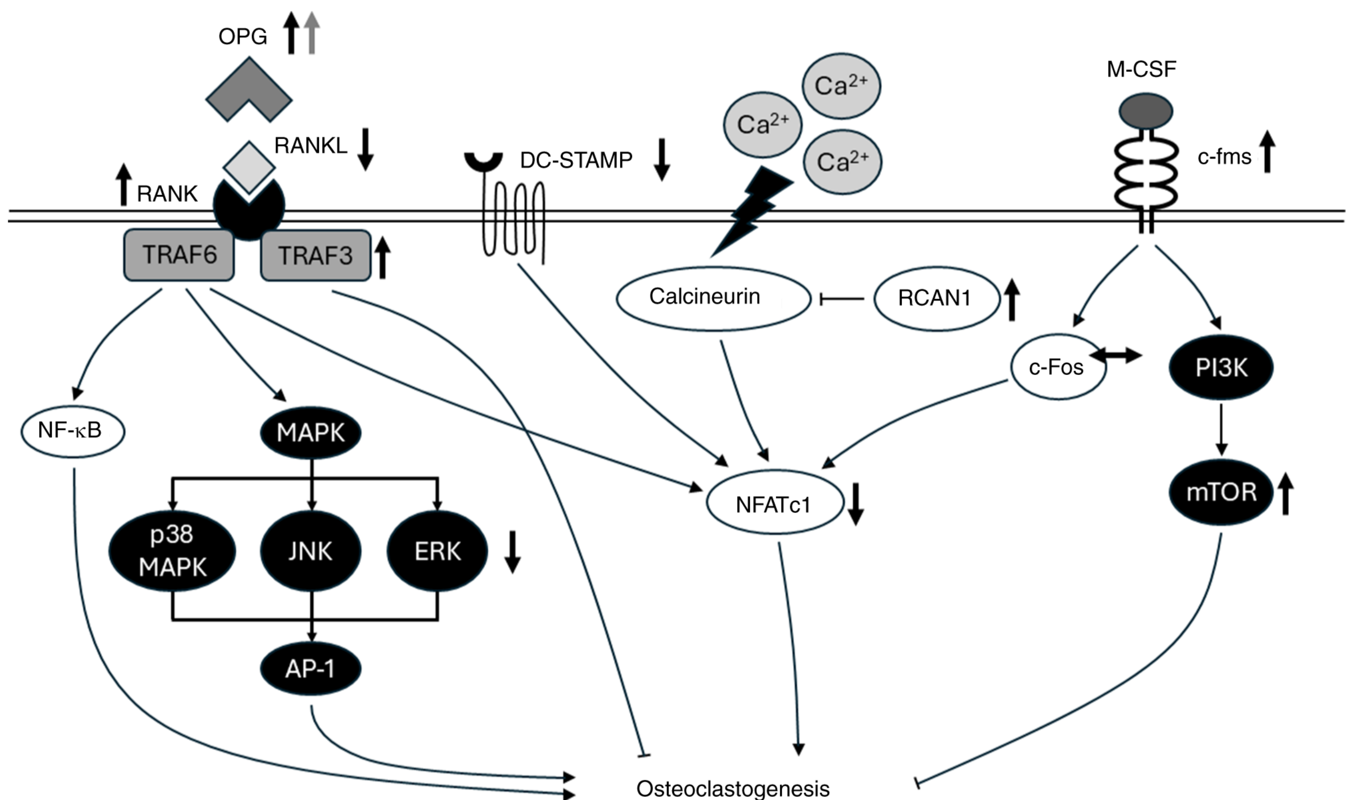

Figure 2. Schematic of the effects of CQ and HCQ on the M-CSF and RANK/RANKL/OPG pathways. The molecular changes caused by CQ and HCQ are represented by black and grey arrows, respectively. AP-1, activating protein-1; c-fms, macrophage colony-stimulating factor receptor; c-Fos, Fos proto-oncogene; CQ, chloroquine; DC-STAMP, dendritic cell-specific transmembrane protein; HCQ, hydroxychloroquine; M-CSF, macrophage colony-stimulating factor; NFATc1, nuclear factor of activated T-cells cytoplasmic 1; OPG, osteoprotegerin; RANK, receptor activator of nuclear factor κ-B; RANKL, receptor activator of nuclear factor κ-B ligand; RCAN1, regulator of calcineurin; TRAF3, tumour necrosis factor receptor-associated factor 3; TRAF6, tumour necrosis factor receptor-associated factor 6; ↑, increase/upregulate; ↓, decrease/downregulate; ↔, no change.

of CQ (10 μM) with OPG further reduced osteoclast formation and activity compared with CQ alone (57). However, this combination treatment resulted in autophagy inhibition to a lower extent, evidenced by a reduction in the number of autophagosomes, LC3II/I ratio and Atg12 levels, whereas the expression level of Beclin 1 and p62/SQSTM1 was increased (57). In mTOR signalling, the levels of phosphorylated mTOR and Raptor were reduced, but the expression levels of G protein β-subunit-like protein (a kinase domain of mTOR that stabilises the interaction between Raptor and mTOR) were increased in M-CSF- and RANKL-induced BMMs treated with CQ and OPG compared with those treated with CQ only. In the AMPK pathway, phosphorylated AMPK was higher; meanwhile, tuberous sclerosis complex 2, Ras homolog enriched in the brain and phosphorylated p70 ribosomal protein S6 kinase were reduced in the CQ and OPG treatment groups compared with the CQ-only group (57). However, Huang *et al* (64) demonstrated that HCQ failed to exert any effect on autophagy in PBMCs isolated from patients with rheumatoid arthritis stimulated by M-CSF and RANKL (64). Therefore, CQ inhibited the autophagolysosomal degradation of TRAF3, thereby forming fewer osteoclasts in response to M-CSF, RANKL and/or LPS. Furthermore, CQ increased the lysosomal pH via the activation of mTOR (a negative regulator of autophagy), thereby inhibiting osteoclastogenesis (57). Compared with CQ, the pharmacological inhibition of autophagy by HCQ was not detected (Fig. 3).

Apoptosis. Apoptosis is a self-destruction process of cells triggered by cellular injury and oxidative stress, which is regulated by two major mechanisms (81). In the mitochondria-dependent (intrinsic) pathway, intracellular stimuli upregulate pro-apoptotic (Bax) but downregulate anti-apoptotic (Bcl-2) molecules, leading to the disruption of mitochondrial outer membrane permeabilization and the release of cytochrome *c*. Subsequently, cytochrome *c* interacts with apoptotic protease activating factor-1, deoxyadenosine triphosphate and procaspase-9, forming an apoptosome, which converts procaspase-9 to caspase-9. The downstream effector caspases (caspase-3 and caspase-7) are activated, acting as a molecular switch for apoptosis (81). In the death receptor-mediated (extrinsic) pathway, Fas ligand and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) are two death ligands recognised by Fas and TRAIL receptors, respectively. Eventually, the Fas-associated death domain and caspase-8 are recruited to form a death-inducing signalling complex and activate downstream effector caspase-3 to induce cell fragmentation and apoptosis (81).

The pro-apoptotic effects of CQ on osteoblasts were observed at higher doses (10-20 μM) but not at a lower dose (5 μM) (41,42,45,76). The levels of Bax, caspase-3 and p53 were increased, whereas the expression levels of Bcl-2 were reduced (42). Treatment with CQ at 50 μM did not cause any change in Bcl-2 levels (43). In the presence of inflammatory

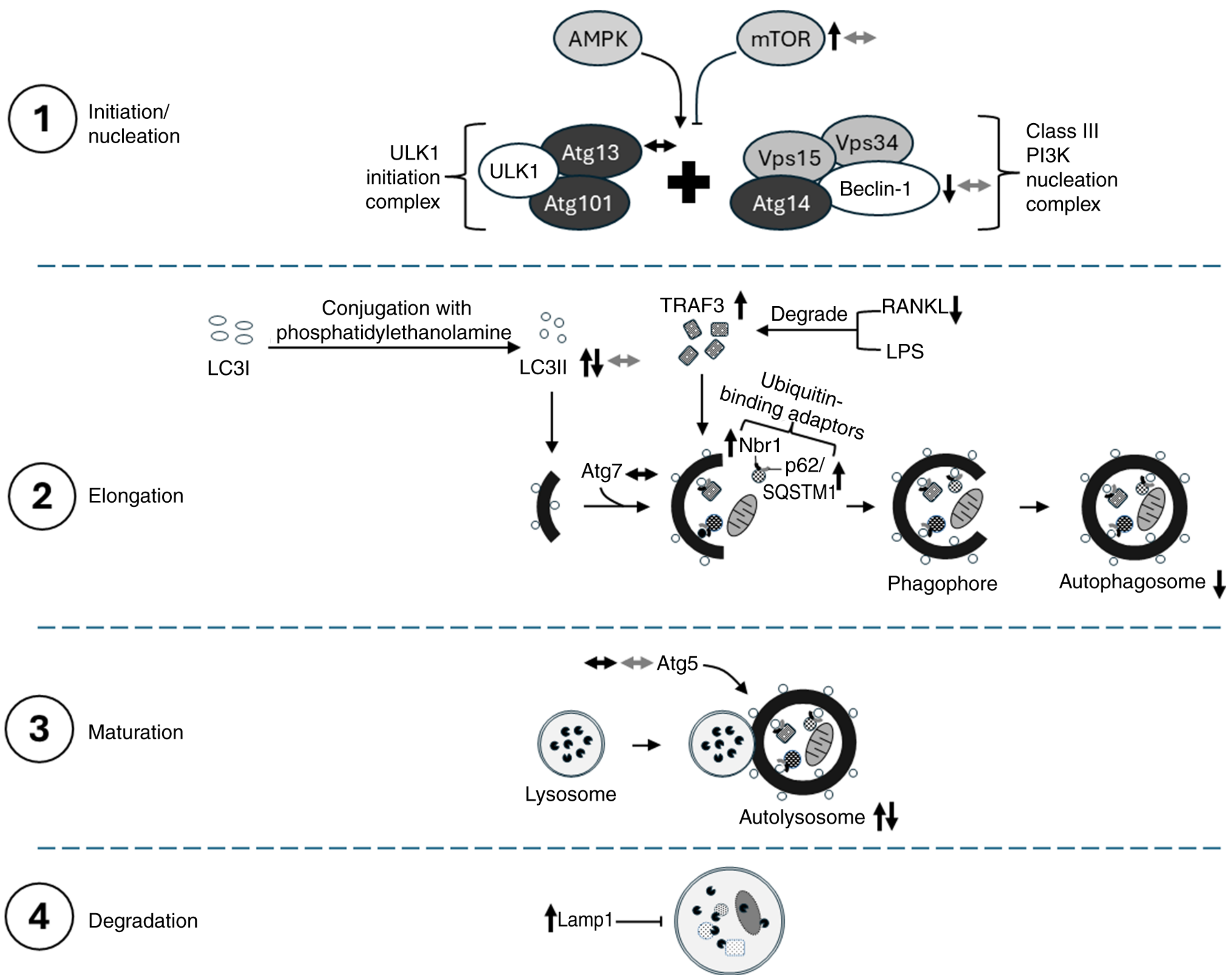

Figure 3. Schematic of the effects of CQ and HCQ on the regulation of autophagy machinery. The molecular changes caused by CQ and HCQ are represented by black and grey arrows, respectively. AMPK, 5'AMP-activated protein kinase; Atg, autophagy-related proteins; CQ, chloroquine; HCQ, hydroxychloroquine; lamp1, lysosome-associated membrane glycoprotein 1; LPS, lipopolysaccharides; Nbr1, neighbour of Brca1 gene; p62/SQSTM1, sequestosome-1; RANKL, receptor activator of nuclear factor κ-B ligand; TRAF3, tumour necrosis factor receptor-associated factor 3; ULK1, Unc-51-like kinases 1; Vps, vacuolar protein sorting; ↑, increase/upregulate; ↓, decrease/downregulate; ↔, no change.

cytokines such as TNF-α, treatment with CQ elevated caspase-3 and cleaved poly (adenosine diphosphate-ribose) polymerases, indicating enhanced osteoblast apoptosis (50,77). The cadmium-mediated apoptotic cell death in rat cranial osteoblasts was also further potentiated by the autophagy inhibitor CQ (51). On the contrary, HCQ (5 µg/ml) demonstrated no effect on apoptotic events in human mesenchymal stromal cells (52).

Several studies have investigated the effects of CQ and HCQ on osteoclast apoptosis. The survival of BMMs stimulated by M-CSF and RANKL was not affected by a lower concentration of CQ, but the number of terminal deoxynucleotidyl transferase dUTP nick-end labelling-positive cells was increased at a higher concentration of CQ (7.5-10 µM) (30). Two studies have reported the differential effects of HCQ on apoptosis using PBMC-sorted monocytes and PBMCs isolated from patients with rheumatoid arthritis stimulated by M-CSF and RANKL, whereby the former reported no apoptotic effects using 5 µg/ml HCQ (16) and the latter indicated an increase in apoptosis with an unknown concentration of HCQ (64). The variation in the treatment dose of HCQ may influence the outcomes observed in these studies.

Oxidative stress. Oxidative stress reflects an imbalance between the production of ROS and reactive nitrogen species (RNS) with the antioxidant defence capacity to detoxify them, inducing lipid peroxidation, protein modifications and deoxyribonucleic acid damage (82). Nicotinamide adenine dinucleotide phosphate oxidase 4 (Nox4) is the main source of ROS production, whereas RNS synthesis is initiated by the interaction between nitric oxide (NO) and superoxide anions (82). SOD, GPx and CAT are antioxidant enzymes that contribute to the first-line antioxidant defence in the body (82). CQ inhibits osteoblastic activity via the induction of oxidative stress. Pretreatment with low-dose CQ (5 µM) had no effect on ROS formation and Nox4 expression in MC3T3-E1 cells (41). A higher concentration of CQ (20 µM) reduced the total antioxidant capacity and SOD levels but increased NO levels,

indicating the elevation of oxidative stress (42). To the best of our knowledge, to date, the antioxidative effect of CQ or HCQ in osteoclasts is limited.

Inflammation. Inflammation refers to the activation of the innate and adaptive immune system that produces an array of inflammatory cytokines, such as TNF-α, ILs, interferons and chemokines (64). These molecules perpetuate inflammation to affect the differentiation and function of osteoblasts and osteoclasts, leading to excessive bone degradation and impaired osteoblast function (64). HCQ administration reduced the generation of inflammatory cytokines in PBMCs isolated from patients with rheumatoid arthritis stimulated by M-CSF and RANKL. The levels of chemokine (C-C motif) ligand 20, CXC motif chemokine ligand 8, IL-1, IL-6, IL-17 and TNF-α, were decreased, but the expression levels of hypoxia-inducible factor 1α remained unchanged (64). Nonetheless, the role of CQ and HCQ in the inflammatory response in osteoblasts remains unclear.

7. Perspectives

In vitro studies have revealed that CQ and HCQ inhibit osteoblastic (28,42-48,52) and osteoclastic (16,23,24,27,30, 34,49,53-58,60-64) activities simultaneously. The inhibition of osteoblast differentiation and mineralisation by CQ and HCQ is mediated through the suppression of autophagy, activation of oxidative stress and osteoblast apoptosis. Autophagy dysfunction increases ROS production and accelerates cell senescence, leading to increased osteoblast apoptosis and decreased numbers of osteocytes (83). In osteoclasts, CQ inhibits osteoclastogenesis via the suppression of the RANK/RANKL/OPG system (49,62,69,70), autophagy (53,54,57,58,64,80) and the inflammatory response (64). Although CQ treatment increases the expression levels of RANK and c-fms (the receptors for RANKL and M-CSF respectively), the OPG/RANKL ratio is increased (69,70), and the expression levels of c-Fos remain unchanged (55,68), thereby leading to the downstream inhibition of ERK and NFATc1 expression during osteoclastogenesis. Furthermore, in osteoclasts, the induction of autophagy contributes to pro-osteoclastogenesis. CQ inhibits autophagy by preventing TRAF3 degradation and limiting RANKL-induced osteoclast formation (27,56). CQ also increases the lysosomal pH, thereby causing the accumulation of mTOR and phosphorylated-mTOR protein to subsequently inhibit autophagy (57,61). However, the inhibition of autophagy by HCQ has not been observed in osteoclasts. In addition, CQ and HCQ enhance osteoclast apoptosis in mature osteoclasts (64).

The inhibition of bone formation and bone resorption by CQ and HCQ occur in parallel, but their net effect on the skeletal system requires further investigation with a study design focusing on the effects of CQ and HCQ on bone health. The selection of the treatment dose, frequency and duration of CQ and HCQ should be carefully considered. Based on the aforementioned studies, the intervention dose of CQ was 2-200 mg/kg in mice and 10 mg/kg in rats (23,24,26-30,33,34,36). Meanwhile, the effective HCQ dose in protecting the bone was 80 mg/kg (37). The calculated human equivalent dose ranged between 9.73 and 973 mg for CQ and was ~389 mg for HCQ in an adult weighing 60 kg. In humans, 200-250 mg CQ or HCQ was used, which was in line with the well-tolerated doses of CQ and HCQ for rheumatic diseases (200-400 mg/day) and novel coronavirus-19 (500 mg/day) (9).

In animals, the short-term continuous daily administration of CQ (<28 days) was revealed to be protective to bone (23,27), and the intermittent administration of CQ was recommended to exert beneficial effects on bone for long-term treatment (24,26). These synergistic effects may not be observed in combination therapy with CQ and other osteoprotective agents (28). Only a paucity of animal studies has investigated the skeletal effects of HCQ (37-40); thus, drawing a concrete conclusion is challenging. The outcomes obtained from preclinical studies were not reflected in the clinical setting. Firstly, the effectiveness of CQ on bone has been widely established *in vitro* and *in vivo*; however, the use of HCQ has been preferred in human studies. HCQ is a metabolite derived from CQ by adding a hydroxyl group, making it less toxic with fewer side effects and allowing it to dissolve more easily in the body (84). Thus, HCQ is a safer medication for patients (84).

Both CQ and HCQ have excellent oral absorption, good bioavailability, high volume of distribution (extensive drug sequestration by tissues), are hepatically metabolized, have a long half-life and their metabolites are excreted through urine and faeces (85). They can cross the placenta but toxicity in the foetus has not been reported (85). Toxicity develops rapidly (1-3 h after ingestion) in the case of overdose (85). The use of CQ and HCQ is associated with several adverse events such as gastrointestinal discomfort, allergic reactions, retinal damage, cardiomyopathy and skin hyperpigmentation (86).

Although CQ and HCQ have protective effects on inflammatory bone loss, the risk of using these agents should not be overlooked. Dose optimisation and regular monitoring are the key for the safe use of CQ and HCQ. Regular ophthalmologic monitoring is important for long-term CQ and HCQ use to prevent retinal toxicity, which can cause irreversible vision loss if not detected early (87). The patient history of ocular disease should be considered before prescribing these medications (87). In addition, regular cardiac monitoring (including electrocardiogram and echocardiography) could be beneficial in detecting cardiac complications early, ensuring the safe prolonged use of these drugs (88). Dose optimisation for CQ and HCQ focuses on balancing therapeutic benefits whilst minimising toxicity. To further minimise risks, intermittent or pulsed dosing strategies (given every other day) may be considered for long-term use. Further research is necessary to confirm the efficacy of intermittent or pulsed dosing in metabolic bone disorders.

Effective bone health monitoring (including diagnostic assessments, regular follow-ups and lifestyle evaluations) is key for the prevention, early detection and management of bone disorders. Dual-energy X-ray absorptiometry is the gold standard for measuring BMD. The assessment is recommended every 1-2 years to monitor bone loss in women >65 years old, men >70 years old and younger individuals with risk factors such as oestrogen deficiency, smoking, low body mass, prior fractures or chronic glucocorticoid use (89). Micro-computed tomography, an advanced imaging

technique that can generate three-dimensional images, enables the high-resolution evaluation of density, geometry and microarchitecture of mineralised tissues (90). The evaluation of bone turnover markers reflects the rate of bone remodelling, which may provide additional information on bone health and the effectiveness of osteoporosis treatments (91). Consistent follow-ups are essential to ensure medication adherence and adequate calcium and vitamin D intake, as well as to address lifestyle factors such as physical activity, smoking and alcohol consumption (92). All these approaches are non-invasive and suitable for human studies. Meanwhile, *in vivo* samples enable the use of bone histomorphometry and a universal testing machine to characterise bone cells, analyse bone microstructure, assess the bone remodelling rate and evaluate bone strength.

The currently available evidence has limitations that need to be addressed. Several of the included studies used CQ and HCQ as inhibitors for autophagy rather than directly investigating the skeletal effects of CQ and HCQ (29,53,66,80). In addition, in certain studies, a statistical test was not performed to compare the CQ or HCQ group and a control group because determining the effect of CQ or HCQ on bone was not the primary objective of the study (28,49,68). Clinical evidence supporting the use of CQ and HCQ for bone health remains limited. The majority of the available data are derived from observational studies in patients with autoimmune diseases such as rheumatoid arthritis and SLE (13-20). Notably, to the best of our knowledge, no large-scale randomised clinical trials have specifically assessed the impact of CQ or HCQ on osteoporosis, fracture prevention or long-term skeletal health. The absence of targeted research makes it difficult to determine their optimal dosing, duration and effectiveness in managing metabolic bone disorders.

The future development trends in the use of CQ and HCQ for bone health may focus on targeted delivery systems, the development of safer analogues and the integration of precision medicine approaches. Various nanotechnology platforms, including liposomes, polymeric nanoparticles, dendrimers, polyelectrolyte complexes, lipid-based nanoparticles and metallic nanoparticles, have been explored to enhance the reformulation of CQ and HCQ (93). These approaches aim to improve target tissue exposure while increasing overall safety and efficacy (93). In addition, developing novel CQ and HCQ analogues through molecular modifications can enhance their pharmacokinetic and pharmacodynamic properties, minimise undesirable side effects and reduce costs (94), while preserving their bone-protective properties. Researchers are also encouraged to explore precision medicine approaches by identifying genetic or metabolic markers to monitor treatment efficacy and determine the patients who would benefit from CQ- and HCQ-based therapies.

While CQ and HCQ show potential for modulating bone metabolism, several gaps in knowledge remain. Further *in vitro* studies are needed to elucidate dose-dependent effects on osteoblasts, osteoclasts and osteocytes, particularly from low to high concentrations of CQ and HCQ. Potential drug interactions and synergistic effects of CQ and HCQ with osteoporosis medications (such as bisphosphonates, denosumab, selective oestrogen receptor modulators, vitamin D and calcium) and natural anti-osteoporotic products should be investigated using cell culture models. Long-term animal studies are necessary to evaluate the effects of chronic CQ and HCQ use on bone microarchitecture, strength and turnover markers. Additionally, the efficacy and safety of optimal dosing regimens (continuous or intermittent administration) and targeted delivery formulations should be thoroughly investigated in animal models before clinical application. In clinical research, large-scale trials with patient stratification should be conducted to evaluate the efficacy and safety profile of CQ and HCQ, as well as to identify genetic and metabolic biomarkers. These trials should include a comparison between CQ and HCQ and standard osteoporosis treatments such as bisphosphonates, denosumab or teriparatide.

8. Conclusion

CQ and HCQ are potentially useful to strengthen the bone. However, the interaction of CQ or HCQ with other compounds may weaken their bone-conserving effects. Therefore, treatment strategies need to be carefully selected for patients with multiple comorbidities. CQ or HCQ are also contraindicated in patients with hepatic and renal insufficiency (85,86). Clinical trials are warranted to determine whether CQ and HCQ can be safely integrated into the management of osteoporosis.

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Availability of data and materials

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Authors' contributions

SKW wrote the manuscript. Data authentication is not applicable. The author read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The author declares that they have no competing interests.

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