DOI: 10.26599/FSHW.2026.9250933

Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: https://www.sciopen.com/journal/2097-0765

China Food Publishing Co.

Biochanin A: An emerging hepatoprotective bioflavonoid

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ABSTRACT: The liver is the largest digestive gland and the core metabolic hub of the human body. Diverse infectious or noninfectious factors can increase the susceptibility of the global population to acute and chronic liver diseases by disrupting the hepatic immune niche. According to statistics, more than 2 million people die from liver diseases each year, and more than 50% of the deaths related to chronic liver disease, liver cirrhosis, and liver cancer mainly originate from the Asia-Pacific region. These staggering figures validate the necessity of developing hepatoprotective agents. Given the cost-effectiveness and multi-target benefits of plant-derived bioactive compounds, natural compounds and their potential derivatives may serve as effective alternative drugs targeting diverse benign and cancerous liver lesions. Among these compounds, biochanin A (BCA), a natural phytoestrogen, is also an isoflavone of food origin with anti-inflammatory and antioxidant properties. It exhibits remarkable pharmacological benefits in alleviating hepatocellular carcinoma, drug-induced liver injury/hepatotoxicity, liver fibrosis, and metabolic dysfunction–associated steatotic liver disease by virtue of its regulatory effects on the cell cycle, apoptosis, autophagy, and glycolipid metabolism. However, BCA has poor bioavailability, and the quality of various dietary supplements containing BCA on the market is inconsistent. Therefore, focusing on the chemical conformation of natural products and application of drug development technology will lay a foundation for the development of clinical hepatoprotective agents and related functional foods.

Keywords: Bioavailability; Biochanin A; Dietary supplement; Liver disease; Mechanism.

1. Introduction

The liver, as a key detoxification and digestive organ in vertebrates, regulates various life activities, such as drug and cholesterol metabolism, gluconeogenesis, glycogen storage, and red blood cell disintegration. It participates in the synthesis of plasma proteins, hormones, and bile acids and recruitment of immune cells[1,2]. Genetic predisposition, virus intervention, and carcinogenic factors are inducing factors that aggravate the deterioration of liver diseases into end-stage organ failure. This situation has caused liver diseases to rank 11th among the top causes of death worldwide. Viral hepatitis, hepatocellular carcinoma (HCC), and liver cirrhosis complications are the main causes of death from liver diseases[3–5]. Therefore, prioritizing the development of hepatoprotective agents is necessary to meet the health management needs of metabolic diseases. The application prospects of natural products in the field of hepatology should receive

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Received 24 January 2025
Received in revised from 6 March 2025
Accepted 3 April 2025

further attention considering that worldwide, approximately 30% of clinical medications originate from natural bioactive substances; single-target drugs often cannot effectively alleviate the progression of liver diseases, which are regulated by multiple signaling molecules; and naturally derived plant compounds exhibit multi-target therapeutic benefits, and therapeutic potential based on the multi-mechanistic targeting of the expression changes of multiple genes[6]. Biochanin A (BCA) is the most abundant isoflavone in chickpea (*Cicer arietinum* L.), with its content reaching as high as 15–150 mg[7]. Therefore, BCA, as a plant-derived isoflavone and functional phytoestrogen, has a high degree of accessibility and can be indirectly supplemented by the daily diet without the need to deliberately obtain it through prescription drugs. In addition, considering that the food-derived compound BCA can bind DNA or proteins, act as an agonist or antagonist of multiple receptors and as a competitive substrate of some enzymes, and exhibit low biotoxicity with good bioactivity, it may become a safe hepatoprotective agent and a functional dietary supplement ingredient[7,8]. This food-derived compound, which has pharmacological activity and nutritional value, reflects the concept of food and medicine homology[9]. The low toxicity of its main functional components in safety evaluations ensures the feasibility of long-term intervention. Moreover, the food and medicine homology therapy is adaptable throughout the entire intervention cycle. It integrates dietary prevention and drug treatment and provides nutritional support during recovery[10].

The liver is abundant in enzyme systems. Although its special anatomical location and hemodynamics provide the necessary metabolic sites for exogenous substances, they also provide a pathological basis for the accumulation of various toxic compounds and thus trigger hepatotoxicity[11]. Hepatotoxins may further attack proteins, lipids, and nucleic acids, thereby increasing serum liver enzyme content and inducing inflammatory infiltration, lipid and protein peroxidation, and DNA damage. This situation is a potential factor driving the progressive deterioration of liver and bile duct function into irreversible and even fatal damage[12]. Although the research and development of hepatoprotective drugs have been prioritized to restore hepatic homeostasis, the healthcare expenditures related to chronic liver disease have exceeded $32 billion, undoubtedly increasing the socioeconomic burden[13,14]. Given that approximately 200,000 plant-derived secondary metabolites have been developed, focusing on the medicinal value of natural products extracted from plants or their artificially modified compounds may yield an economical natural remedy[15]. Previous studies have demonstrated that BCA could act as a potential therapeutic agent against squamous cell carcinoma by targeting the inactivation of nuclear factor-kappa B (NF-κB) subunits p50 and p65[16]. It might exhibit strong therapeutic potential for alleviating myocardial hypertrophy and fibrosis-related cardiac remodeling phenomena by reversing the inductive effects of angiotensin II on cardiac fibroblast proliferation, collagen deposition, and oxidative stress[17]. In addition, in a unilateral ureteral obstruction–induced renal fibrosis mouse model, BCA could exert antioxidant effects by activating the nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) signaling pathway[18]. BCA relieved atherosclerosis by activating the peroxisome proliferator-activated receptor γ (PPARγ)/liver X receptor α and PPARγ/HO-1 signal cascades to induce anticholesterol deposition and anti-inflammatory

effects[19]. Therefore, botanists and researchers believe that BCA may be a potential hepatoprotective agent because of its pharmacological properties, such as anticancer, anti-inflammatory, antioxidant, antilipid, and anticollagen fiber deposition properties. In addition, because dietary polyphenol supplements rely on the synergistic effect of their diverse bioactive components to show considerable therapeutic benefits in alleviating the progression of metabolic dysfunction–associated steatotic liver disease (MASLD), the nutritional value of numerous dietary supplements on the market seems to be sublimated in the subjective consciousness of consumers[20]. However, according to the data of the Drug-Induced Liver Injury Network Registry, approximately 20% of adult liver injuries are induced by over-the-counter herbal and dietary supplements. This contradictory statement may be ascribed to the limitations of market supervision. That is, the application of functional supplements and herbal preparations is considered to be harmless by default, and the safety of these products does not need to be repeatedly verified by manufacturers[21]. Therefore, this review not only emphasizes the potential of BCA for the prevention and treatment of liver diseases, but advocates focusing on the effective development of dietary supplements rich in BCA and other isoflavones on the market and the standardization of product information. Moreover, it presents feasible strategies to improve the bioavailability of BCA from the aspects of drug development engineering.

2. Overview of BCA

2.1. Source

BCA is a methoxylated isoflavone that can be isolated from edible plants, such as chickpeas, red clover (*Trifolium pratense* L.), peanut (*Arachis hypogeae* L.), soybean (*Glycine max* L.), alfalfa (*Medicago sativa* L.), and *Astragalus* (*Astragalus membranaceus* Bunge)[22]. It is mainly enriched in the leaves of red clover, whereas its available extraction amount in peanuts, alfalfa, and *Astragalus* is generally low[23]. In addition, BCA widely exists in the human daily diet and is naturally distributed in fruits, vegetables, nuts, tea, and red wine[24]. The use of macroporous resins is a low-cost and high-yield method for extracting BCA, with the yield and purity of the extracted BCA reaching 87.13% and 95% respectively. However, updated, efficient, and economic extraction technologies are still needed and can be developed by selecting appropriate mobile phase solvents and ratios, switching to applicable chromatographic columns, or applying high-performance liquid chromatography (HPLC)[23].

2.2. Molecular structure

BCA, an isoflavone in the flavonoid family, is chemically named 5,7-dihydroxy-4′-methoxy isoflavone. It exhibits a certain lipophilicity, molecular weight of 284.267 g/mol, molecular formula of $C_{16}H_{12}O_5$, and melting point of 210 ℃–213℃; it often exists in the form of a white crystalline powder and is soluble in ethanol and dimethyl sulfoxide[7,25] (**Fig. 1**). Its antioxidant and anticancer properties are partly dependent on the specificity of its chemical structure. Ketone groups and the position and number of hydroxyl groups attached to rings A and B are prerequisites for its antioxidant activity. In addition, the hydroxyl group on C7 of ring A, the 2,3-double bond structure on ring C, and the inclination of the methoxy group in BCA to

absorb electrons when connected to the benzene ring markedly increase its antioxidant potential. The compound derived by esterifying the carbon 7-position in ring A of BCA showed significant resistance in the proliferation of MCF-7 and Ishikawa cells, implying that the good anticancer properties of BCA are endowed by the 7-position structure of its ring[23]. BCA has a topological polar surface area of approximately 79.90 $Å^2$, suggesting that it can effectively prevent the growth of MCF-7 breast cancer cells by inhibiting aromatase activity[7].

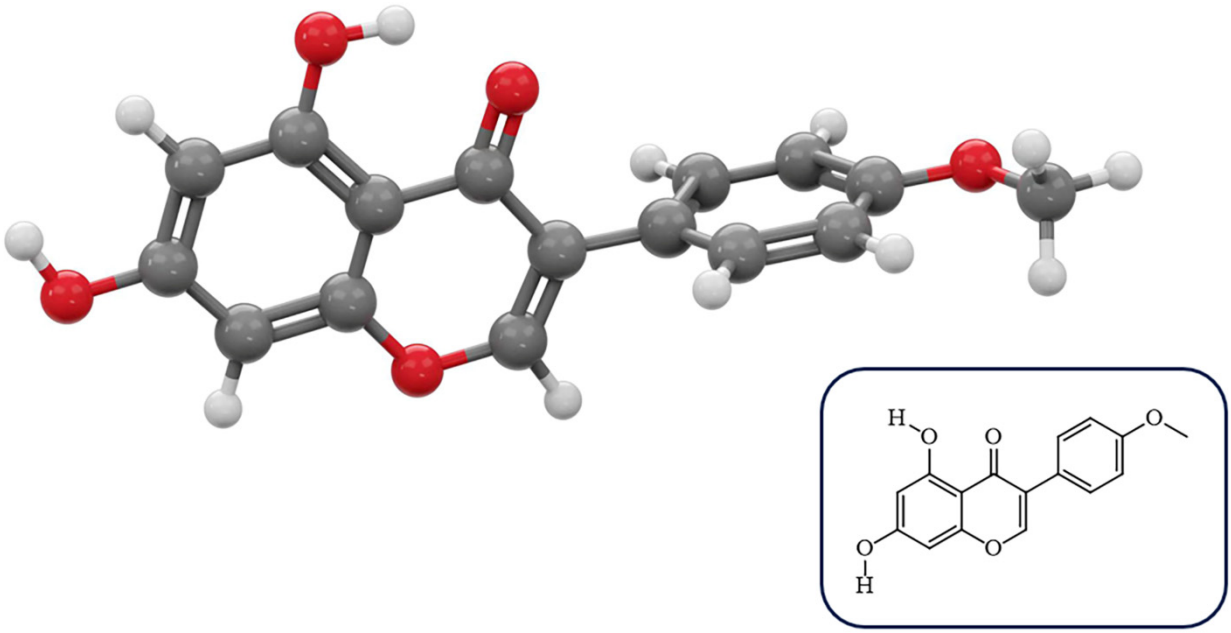

Fig. 1. Molecular structure of BCA ($C_{16}H_{12}O_5$).

2.3. Function

BCA can act as a neuroprotective agent, immunomodulator, vasodilator, and anti-inflammatory and antioxidant active ingredient, demonstrating high-sensitivity chemopreventive potential and conferring considerable resistance against hormone-dependent cancers, atherosclerosis, diabetes, osteoporosis, bacterial or pathogenic microorganism infections, and arthritis; moreover, it can maintain endothelial integrity and biological functions[23,26–29]. BCA, an estrogen receptor β (ERβ)–selective isoflavone, can be regarded as a nonsteroidal estrogen with estrogenic effects and simultaneously executes estrogenic and antiestrogenic bioactivities given its structural similarity to 17β-estradiol, the most potent mammalian estrogen, and affinity to ERs[30]. More than 20 isoflavone compounds have been extracted and identified from red clover. Among these compounds, isoflavones with phytoestrogen effects are increasingly valued in applications for ameliorating postmenopausal diseases and estrogen deficiency–related symptoms because in countries with a high proportion of dietary isoflavone intake, the prevalence of estrogen deficiency–related diseases and symptoms is markedly inhibited. Therefore, the estrogen-promoting isoflavone BCA derived from red clover extract may be suitable as a dietary supplement for relieving menopausal symptoms in women[31]. Notably, under the chronic stimulation of conventional hormone therapy, menopausal women show increased susceptibility to breast cancer, heart disease, stroke, and blood clots. Therefore, red clover isoflavones may

replace hormone therapy and become a safe dietary health product. Red clover dietary supplements contain various isoflavone compounds, including BCA, which may be present at levels of up to 1,350 mg/100 g[32].

Given the above information, further focusing on the optimization of the bioavailability and drug metabolism patterns of BCA on the basis of the analysis of its chemical conformation or structure–activity relationships, will be conducive for exploring its massive unknown therapeutic potential and thus enhance its consumption value in the food and medical markets.

2.4. Toxicity

Flavonoid compounds can be isolated from fruits, vegetables, and plant-derived beverages, and the accessibility of their sources in the daily diet may verify that their biotoxicity is low[33]. In addition, herbal active substances have been included in the ingredient lists of numerous dietary supplements and are widely sold on the market. Therefore, their safety and effectiveness do not need to be repeatedly verified by the US Food and Drug Administration (FDA) or developers[34]. BCA, as a main flavonoid component in a marketed red clover extract (PureWorld Botanicals, South Hackensack, NJ), may be harmless considering that its content in the extract is as high as 14.5%[35]. An oral toxicity study on mice subjected to subacute BCA (3/10/30 mg/kg) exposure for 28 days reported that BCA resulted in no remarkable toxic effects on general clinical signs. The body and organ weights and hematological parameter indicators, including red and white blood cell counts, of the mice did not show abnormal fluctuations related to BCA administration. In addition, the activities of tissue damage markers, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase–MB isoenzyme, and lactate dehydrogenase (LDH), and oxidative stress parameters, including superoxide dismutase (SOD), glutathione (GSH), catalase (CAT), and malondialdehyde (MDA), did not significantly differ between mice treated with BCA and the control group. No abnormal changes in lipid profiles and morphological parameters related to the liver, kidneys, heart, and lungs were found[36]. Not only that, a 28-day repeated-dose subacute toxicity test of a novel BCA–chromium (Cr) (III) complex conducted in KM mice showed that oral gavage at a dose of 1.5 g/kg/d did not cause death in mice. No abnormalities were observed in the mice's blood routine, liver and kidney functions, pancreatic tissue, glycolipid metabolism, antioxidant capacity, behavior, fur, feces, urine, secretions, and mental state. Moreover, there was no significant fluctuation in the mice's body weight, indicating that the complex has no significant subacute toxicity to the digestive system. Therefore, the organic Cr complex obtained by chelating Cr (III) with the ligand BCA not only ensures good hypoglycemic activity in db/db mice and C57 mice, but also the chelation with the low-toxicity ligand lays a foundation for the development of safe functional Cr-containing foods or pharmacologically active Cr complexes[37]. However, the structural similarity between BCA and estrogen and the certain affinity of BCA to ERs increases doubts about whether its estrogenic effect is accompanied with certain unknown risks. Innate genetics, acquired dietary factors, and hormone secretion may all interfere with the effect of BCA in the body. This situation also implies that BCA results in different tolerances and curative effects in different individuals. However, for the general population, supplementing an appropriate amount of plant-derived

BCA does not cause substantial or irreversible functional damage to the body. Certain uncontrollable risks arise, especially in individuals with a history of hormone-related cancer or hormonal imbalance, only when extremely high doses or concentrated forms of BCA are ingested[7]. Notably, most of the studies that currently assert BCA as a low-toxicity compound are based on oral toxicity tests in mice, mainly focusing on exposure to subacute toxicity doses, and do not involve toxicity detection under intraperitoneal or intravenous injection conditions. Considering the need to explore the administration mode for improving BCA's bioavailability, it is necessary to further conduct acute/chronic and subacute/subchronic toxicity tests targeting different administration routes. Furthermore, human clinical toxicity data related to BCA is scarce. Species differences dictate that the safe dose range determined in mouse models cannot be directly extrapolated to primates. Therefore, conducting large-scale clinical toxicity studies to clarify the adverse reactions and tolerability under high-dose BCA intervention is of great reference significance.

2.5. Pharmacokinetics

2.5.1. Absorption

BCA not only has a considerable permeation effect on the skin, intestine, and kidney cells, but is also a potential drug capable of crossing the blood–brain barrier and penetrating brain tissue[7]. After being ingested, BCA often conjugates with sugar in the body to form glycosides, with sissotrin being its glycoside form[23,38]. Glycosides exhibit limited absorption in the upper gastrointestinal tract because of their high molecular weight. They often pass through the small intestine to the colon, where β-glucosidase derived from *Lactobacillus* and *Bifidobacterium* strains catalyze their hydrolysis into aglycones for absorption by the body[39]. Among them, Gut *Enterococcus* sp. MRG-2 and *Lactococcus* sp. MRG-IF-4 are responsible for the hydrolysis of sissotrin into free BCA[38]. The aglycones absorbed by the intestine through passive diffusion also undergo extensive metabolic transformation through reduction or demethylation reactions or interact with ERs to exert bioactivity[23,39]. This phenomenon suggests that because the gut microbiome of each individual is highly personalized, the concentrations of BCA detected in plasma and urine remain highly differentiated even if the same amount of the compound is ingested orally[40].

BCA could smoothly penetrate the enterocyte membrane in a rat intestinal perfusion model and quickly penetrate Caco-2 cells for effective absorption because of its considerable membrane permeability[41]. Although BCA exhibits good permeability across cell membranes, the intestinal absorption of BCA is adversely affected by the high rate of intestinal metabolism to conjugated metabolites and the potential excretion of BCA back into the intestine by ATP binding cassette proteins located in the apical membrane of intestinal cells. The regulation of BCA excretion by ATP binding cassette transporters via the biliary pathway and the enterohepatic recirculation of BCA mediated by the conjugation or hydrolysis of enzymes and gut microbiota subsequently will also determine the ultimate fate of BCA[33].

2.5.2. Distribution

After the intravenous injection of BCA into rats, its distribution volume in the body shows a higher trend because of its hydrophobicity[33]. The biotransformation pattern of BCA involves multiple organs,

with the detectable concentration of BCA being lowest in the blood (0.96%); lower in bile (1.38%); and highest in urine (45.10%)[23]. In terms of tissue distribution, BCA shows the highest distribution amount in the rat liver[42]. However, isoflavone metabolites are partially distributed in urine and feces. Hepatic enzymes and gut microbiota have biological characteristics of intervening in drug metabolism, implying that the liver is not the only site of isoflavonoid metabolism. The intestinal tract, and target tissues sensitive to estrogen-induced carcinogenesis may intervene in the extrahepatic metabolism of BCA. Therefore, the content of BCA in extrahepatic kidney tissue is second only to that in the liver, whereas those in the lungs, spleen, heart, and brain are low and decrease in that order. This distribution pattern may be explained by the fact that the liver and kidneys are the main organs responsible for metabolic and excretory functions. Therefore, the distribution of BCA in the liver and kidneys far exceeds those in other tissues[42].

2.5.3. Metabolism

The isoflavone genistein (GEN), BCA conjugates, and GEN conjugates are the main metabolites after the biotransformation of BCA[27]. BCA, a 4-*O*-methyl derivative of GEN, can be *O*-demethylated into GEN or hydroxylated under the mediation of cytochrome P450 (CYP450) enzymes[33]. The *O*-demethylation of phytoestrogens mainly occurs in the liver, and BCA derived from chickpeas and red clover is metabolized and derived into GEN with stronger estrogenic activity under the mediation of human liver microsomal enzymes. Secondly, after hydroxylation in human or rat liver microsomes, BCA is converted into 3′-, 6- or 8-hydroxy BCA or GEN. The in vivo effects of these hydroxylated metabolites still need further study[33]. Notably, *Eubacterium limosum*, a strict anaerobe with *O*-demethylation activity isolated from the human intestinal tract, also mediates the demethylation of BCA into GEN[28]. BCA and its metabolite GEN have been developed in batches and consumed as active ingredients in isoflavone herbal supplements. Their chemopreventive properties and efficacy in treating cardiovascular diseases and delaying menopausal symptoms have rich research value. Therefore, focusing on the pharmacokinetics of BCA may lay the foundation for quantifying the concentration–effect relationships of more potential therapeutic activities of BCA[27].

While the strong permeability of BCA contributes to its metabolic characteristic of rapid absorption, its extensive first-pass metabolism and biliary elimination pathways limit its in vivo bioavailability. After the intravenous administration of BCA, its plasma concentration decreases biexponentially because it is rapidly metabolized[27]. Glucuronidation, sulfonation, and methylation are the main biological processes involved in the phase II metabolism of BCA, whereas oxidation, reduction, and internal hydrolysis may constitute main phase I reactions[28]. Among these reactions, glucuronidation is the main conjugation reaction of BCA in the rat liver and intestinal microsomal preparations, and the glucuronide conjugates derived from BCA may be 7-OH and 5-OH glucuronic acid derivatives[27]. BCA exhibits a high distribution volume and metabolic clearance rate in rats because it is derived into glucuronide and sulfate conjugates after extensive metabolism in the intestine and liver. However, methylation at the 7-position in the structure of BCA may suppress glucuronidation/sulfation. It is worth noting that although the methylation degree of flavonoids is

significantly positively correlated with their metabolic stability, the excessive methylation of BCA may inhibit its binding to ERs, thereby weakening its bioactivity[33].

Ultrahigh-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry was used to characterize the in vivo and in vitro metabolism of BCA. It identified 43 BCA metabolites in rats, as well as 22 BCA metabolites derived from liver microsomes and 18 BCA metabolites in intestinal flora. The metabolic rates of BCA in liver microsomes and intestinal flora were extremely close and were 89.30% and 89.67% respectively. BCA is metabolized into 45 metabolites under the mediation of 23 metabolic pathways. Glucuronide conjugation and sulfate conjugation are the main metabolic forms of BCA in the blood. Moreover, GEN and the glucuronide and sulfate conjugates of BCA have been detected in the blood. BCA mainly undergoes glucuronidation, demethylation, and sulfonation in bile; oxidation and glucuronidation in urine; and hydrogenation in feces. Notably, the content of BCA metabolites in intestinal flora is relatively low[28].

2.5.4. Excretion

The main organ excreting isoflavonoids is generally believed to be the kidney, and the biliary and fecal pathways also mediate part of isoflavonoid excretion[43]. After sulfonation or glucuronidation by phase II enzymes, isoflavone conjugates are excreted into the bile and further reabsorbed by the body after deconjugation by gut microbiota[32]. Notably, the recovery rate of BCA from the bile is approximately 18.8% after treatment of bile samples with sulfatase/glucuronidase[27]. In short, after absorption through the intestine, isoflavonoids enter the portal vein and reach the liver with the blood flow and are subsequently enzymatically conjugated and excreted in the bile. They may be hydrolyzed under the mediation of gut microbiota or even reabsorbed. However, BCA glucuronide, which is subordinate to flavonoid conjugates, may be an exception, and direct intestinal excretion may be a more effective metabolic pathway for BCA glucuronide than the biliary pathway. This particular excretion mode for a flavonoid glucuronide, that is, the direct intestinal excretion pathway from plasma, has opened up considerable application prospects for BCA recovery strategies based on enteric recycling[44].

2.6. Bioavailability

2.6.1. The poor bioavailability of BCA

Although BCA is a naturally occurring isoflavone of dietary origin and a bioactive ingredient with anticancer effects, its solubility in water is only 7 μg/mL[41]. Its oral bioavailability is limited to 4.6% because of its susceptibility to hydrophilic degradation in the gastrointestinal tract and remarkable first- and second-pass metabolism, weakening its potential as a chemotherapy drug[30]. BCA is considered as a Biopharmaceutics Classification System Class II drug because its dissolution rate can inversely inhibit its absorption rate[22]. Secondly, flavonoids are often involved in enteric and enterohepatic recirculation and extensive metabolic pathways. Such an involvement leads to the obstruction of their entry into the systemic circulation. Therefore, their bioavailability is generally low[41]. In addition, the high metabolic clearance rate of BCA is a key factor that places its bioavailability at a disadvantage[25]. Notably, a study reported

that under oral conditions in Sprague–Dawley rats, the bioavailability rates of 5 and 50 mg/kg BCA were 2.6% and 1.2%, respectively. However, the bioavailability of 5 and 50 mg/kg BCA injected intraperitoneally reached 40% and 23%, respectively. The maximum plasma concentration after the intraperitoneal injection of 50 mg/kg BCA was 80 times higher than that after oral administration. This finding indicates that maintaining the in vivo activity of BCA may need to rely on intraperitoneal administration[27].

Flavonoids are low-molecular-weight plant polyphenolic compounds derived through photosynthesis, and glucuronidation, poor solubility and stability are often the main factors that prevent phenolic compounds from achieving their ideal bioavailability[30,45]. Among them, isoflavones are composed of large planar conjugated systems with short intermolecular distances and tight structures, which invisibly increase the challenge of dispersing their molecular structures by solvents and ultimately contribute to their limited water solubility and bioavailability[46,47]. However, the ester derivatives of bioactive phenols, such as the carbamate ester derivatives of BCA, have greater bioavailability and metabolic stability than their parent compounds. Furthermore, the development of novel delivery systems, modifications of chemical structures, application of nanoformulations, and encapsulation of drugs based on liposomes or nanoparticles may all be beneficial to improve the stability and bioavailability of BCA.

2.6.2. Strategies for improving the bioavailability of BCA

Relevant studies first increased the content of isoflavones in chickpea through germination and then synthesized chickpea sprout extract/β-cyclodextrin inclusion complexes. The preparation of chickpea sprout extract-based inclusion complexes might result in the faster tissue absorption rate and moderate metabolic rate of BCA. Moreover, the multiple hydrophilic hydroxyl group of β-cyclodextrin endowed inclusion complexes with increased wettability and solubility, thereby improving the ability of the drug molecules to permeate cell membranes[42,48]; a novel BCA–nicotinamide cocrystal was designed and developed. The solubility and drug release performance of the BCA–nicotinamide cocrystal had significantly improved compared with those of free BCA. The cocrystal further promoted the accumulation of BCA in pancreatic tissues[49]; technological improvements in dissolving, encapsulating, entrapping, or attaching BCA onto microparticle matrices have enabled the application of enteric-coated microparticles as a direct intestinal drug delivery system. The enteric-coated microparticles of BCA achieved sustained drug release, and their oral bioavailability had significantly increased by six times compared with that of pure BCA[30]; Wang et al. developed an oral nanostructured lipid carrier formulation rich in BCA. As a result of the inclusion of liquid lipids, the matrix of BCA–nanostructured lipid carrier was in a disordered state, which would be conducive to promoting stable drug storage performance and improving drug loading capacity[50]; solid dispersions (SDs), as hydrophilic carrier systems, can not only exert their own solubilization effects, but might further enhance the solubility of BCA by inducing BCA to transition from its crystalline form to its amorphous form[51,52]. SDs prepared with polyethylene glycol 660 hydroxystearate (Solutol®HS15) and hydroxypropylmethylcellulose (HPMC 2910) effectively improved the bioavailability of the poorly soluble flavonoid BCA. As the proportion of the surface active hydrophilic carrier Solutol®HS15 in the SDs

increased, the solubility of BCA accordingly increased by 8–60 times[51]; preparing BCA-loaded micelles with Pluronic F127 and Plasdone S630 remarkably optimized the water solubility of BCA and its permeability through the Caco-2 cell monolayer. Furthermore, BCA in the mixed micelles transitioned from an initial explosive release to sustained release. The relative oral bioavailability of BCA-loaded micelles with Pluronic F127 and Plasdone S630 significantly increased by 2.16 times compared with that of free BCA[53]; developing BCA-containing oromucosal film formulations on the basis of solvent casting may be a simple alternative strategy that does not rely on chemical structural modification[41]. The formulated films markedly increased the dissolution rate of BCA. This effect was closely related to the solubilization effect of hydrophilic additives and the involvement of amorphous BCA. Moreover, BCA released from the film preparation could directly penetrate porcine buccal tissue without undergoing first-pass metabolic conversion into GEN[41]; ERs tend to localize in deep dermal tissue, and cyclodextrin can serve as a biocompatible inducer that promotes the penetration of active molecules into the skin. Therefore, the ability of BCA to penetrate the epidermis and dermis can be remarkably enhanced by incorporating hydroxypropyl-β-cyclodextrin into the hydrophilic base hydroxypropyl methylcellulose hydrogel[54,55]; focusing on the synthesis of novel drug carriers, such as poly(*N*-isopropylacrylamide [NIPAM]-co-acrylic acid), p(NIPAM-co-acrylic acid [AA]), copolymer prepared through the free radical polymerization of NIPAM monomer with 5 mol% AA and 1.5 mol% ethylene glycol dimethacrylate as a cross-linker, is necessary to optimize the physicochemical properties of the poorly soluble isoflavone BCA[22]. The carboxyl and amino structures in the copolymer p(NIPAM-co-AA) confer the potential to control drug release. Considering poly(*N*-isopropylacrylamide-co-acrylic acid), p(NIPAM-co-AA), copolymer having a dual response to temperature and pH, its use as a sustained-release carrier for BCA might contribute to the modified release of BCA in the acidic vaginal environment and the rectal weakly alkaline environment[22]; Jadav et al. synthesized a novel thiolated xanthan gum (XG)–stearylamine bioconjugate through functionalizing the polysaccharide XG to prepare mucoadhesive nanoparticles for delivering BCA. During nanoparticle preparation, XG underwent thiolation by cysteamine hydrochloride to enhance mucosal adhesion, and the lipid component stearylamine was conjugated with XG to promote the effective encapsulation of the loaded hydrophobic BCA. This bioconjugate induced the cytotoxic effect of BCA against human epidermoid cancer cells and effectively improved the storage stability, sustained release ability, and drug entrapment efficiency of BCA[56]. In summary, by drawing on the above-mentioned formulation preparations or delivery carriers, it may have considerable reference significance for optimizing the hepatic delivery of BCA.

3. The protective mechanisms of BCA against HCC

3.1. Hepatocellular carcinoma

HCC is the pathological phenotype of approximately 75%–85% of primary liver cancers. HCC, as the most invasive and most commonly diagnosed malignant liver tumor, is the third leading cause of cancer deaths worldwide[57–59]. Global epidemiological data show that approximately more than 2 million people

die of liver diseases every year, and the deaths of 600,000–900,000 people can be attributed to liver cancer[4]. Hepatitis C, followed by alcohol and MASLD, is the leading cause of HCC in Western countries, whereas hepatitis B is the main risk factor for HCC in Asia and Africa[60]. HCC is a cancerous lesion involving multiple signal cascades. Hepatitis B virus (HBV) and HCV infections can further initiate chronic inflammatory responses through viral replication, proinflammatory cytokine secretion, and immune cell recruitment, with inflammatory infiltration becoming the driving force inducing tumor cell proliferation and immune escape[61]. In addition, pathological changes in the expression of proto-oncogenes; tumor suppressor genes; and multiple genes regulating cell proliferation, apoptosis, and differentiation are key mediators that exacerbate HCC[62]. Given that relevant studies on multiple carcinogenic mechanisms and multi-signal cascades regulating tumor cell proliferation, differentiation, and invasion are constantly being updated, the potential of plant-derived molecular targeted anticancer drugs in interfering with HCC has gradually attracted the extensive attention of scientists[63]. Sorafenib (SOR) is a classic anti-HCC drug recognized by the US FDA. However, the variable network of tumor signals in series in the liver indicates that the tumor microenvironment is complex. Relying on the therapeutic activity of single-target drugs may not meet accurate anticancer needs, and the synergistic effects of multiple anticancer drugs or multi-target drugs may be required to effectively alleviate the carcinogenic effects driven by multiple signal cascades[57,64]. Notably, the bioactive ingredients in approximately two-thirds of anticancer drugs are isolated from natural products[65]. Among them, the food-derived natural compound BCA can show good bioactivity in inhibiting cancer cell proliferation and invasion, synergistically enhancing the efficacy of chemotherapy drugs and promoting cancer cell apoptosis by virtue of its multi-target treatment benefits. Herein, we focus on highlighting the potential of BCA as a highly promising candidate for chemoprevention and adjuvant therapy in HCC.

3.2. Promoting cell cycle arrest

As a classic mitogen-activated protein kinase (MAPK) signal cascade involved in cell proliferation and survival, the overexpression or mutation of proteins in the rapidly accelerated fibrosarcoma (RAF)–mitogen-activated protein kinase kinase–extracellular signal–regulated kinase (ERK) pathway, is often an endogenous factor that induces the further carcinogenesis of tumor cells[66]. However, compared with mitogen-activated protein kinase kinase inhibitors, RAF kinase inhibitors can inhibit ERK signal transduction more significantly and exhibit superior anticancer activity in clinical practice[57]. SB590885, a selective inhibitor of the serine/threonine–protein kinase v-Raf murine sarcoma viral oncogene homolog B1, has been studied. The combined administration of BCA and SB590885 could synergistically inhibit the proliferation of HCC cells and promote G0/G1 phase cell cycle arrest. The expression of cyclin D1, a protein factor regulating the transition from the G0/G1 phase to the S phase, was down-regulated after combined intervention[57].

While surgical intervention can remove local tumors, the five-year survival rate of patients with HCC continues to range from only 20 to 51%. In addition, although the antiangiogenic multi-kinase inhibitor SOR

is an approved anti-HCC chemotherapy drug, its long-term use leads to the drug resistance of cancer cells. Eventually, it can only extend the median overall survival of patients with HCC by approximately three months[57]. Therefore, strengthening the anticancer effect of targeting multiple genes and multiple pathways through assistance with plant-derived natural anticancer agents may improve the sensitivity of SOR in targeting cancer cells[67,68]. Soy isoflavones, as bioactive chemopreventive agents, may be natural compounds that synergistically enhance the anticancer activity of SOR and prolong the survival of patients with HCC[69]. Although the single administration of SOR or the soy isoflavone component BCA could inhibit cell cycle transition from the G0/G1 phase to the S phase, BCA could further enhance the effect of SOR in negatively regulating cyclin D1 when concurrently administered[70].

3.3. Promoting apoptosis

Cell cycle arrest can further induce apoptosis-promoting reactions. Therefore, the coadministration of BCA with SOR, BCA with SB590885 would further initiate the overexpression of the apoptosis-promoting protein Bax and inhibit the activity of the antimitochondrial apoptosis factor Bcl-2. That is, coadministration could up-regulate Bax/Bcl-2; such an up-regulation could indicate that the apoptosis of HCC cells is remarkably promoted[57,70]. The downstream initiators involved in the mitochondrial apoptotic signaling cascade include caspase-9. Activated caspase-9 can further trigger the overexpression of executioner caspases, including caspase-3. BCA and SOR synergistically enhanced the activity of caspase-9 and caspase-3, further corroborating the possibility of promoting mitochondrial apoptosis through coadministration[70–72]. Notably, the BCA-induced apoptosis of human hepatoma HuH-7 cells was also related to the up-regulation of caspase-3 expression[73]. Furthermore, isoflavones, depending on their estrogenic and antiestrogenic effects and their inhibitory effects on DNA topoisomerase, tyrosine kinase activity, and angiogenesis, can serve as natural antitumor compounds[74,75]. Among them, the bioactive component BCA of soy-derived isoflavones interfered with the growth of the human hepatoma cell lines HepG2, Hep3B, HuH-7, PLC, and HA22T in a dose-dependent manner. Moreover, it triggered tumor cell death mediated by the proapoptotic effect by activating caspase-3 and promoting the inactivation of the antiapoptotic proteins Bcl-2 and Bcl-X_L, and the tyrosine dephosphorylation of cellular proteins in human hepatoma cells[76]. Not only that, cell apoptosis and necrosis are often accompanied with cell lytic damage, which leads to the leakage of LDH from the cytoplasm to the extracellular space. The activity of extracellular LDH can be considered as a functional indicator for evaluating the integrity of cell membranes and cytotoxicity[73]. BCA could enhance toxic effects against human hepatoma HuH-7 cells by inducing the overexpression of LDH. Therefore, BCA is a natural targeted therapeutic agent for inducing the apoptosis of human hepatoma cells[73].

3.4. Inhibiting proliferation

The phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) is a key signaling cascade involved in regulating the proliferation of HCC cells. The PI3K pathway is overactivated in 30%–50% of patients with HCC[57]. However, the coadministration of BCA and

SB590885 further enhanced tumor suppressive activity by inhibiting ERK MAPK, and PI3K/AKT/mTOR signaling and promoting the inactivation of proliferating cell nuclear antigen (PCNA), a tumor proliferation marker[57]. Given that the coadministration of BCA and SB590885 did not cause toxic damage to the liver and kidneys, the two therapeutic agents may be used as safe anticancer candidate drugs in combination in clinical practice to reduce the lethality of HCC[57]. Furthermore, Ki-67 is present at high nuclear levels in proliferating cells. It can serve as a proliferative marker for regulating neoplastic proliferation. However, SOR and BCA could synergistically resist Ki-67 overexpression[70,77]. In conclusion, the coadministration of BCA and SOR would induce cell cycle arrest and promote mitochondrial apoptosis, thereby finally exerting a cytotoxic effect of synergistically inhibiting cancer cell proliferation in the human HCC cell line HepG2[70].

3.5. Exerting phytoestrogenic activity

Miso and/or soy sauce produced through fermentation from soybeans, rice, wheat, or oats are generally regarded as a traditional seasoning or food in daily diet structures. Among them, BCA derived from bean products is also classified as a dietary component of miso[78]. In mice, soy protein isolate consumption has been proven to prevent diethylnitrosamine-induced liver tumors[79]. Therefore, miso may also be a potential active substance against liver tumors in mice independent of cytotoxicity, and its antihepatoma activity can be enhanced by the synergistic effect of simultaneously mixing BCA, protease inhibitors, and diverse fermenting enzymes[78]. Research showed that feeding mice with 10% miso or 20 ppm BCA decreased liver tumor multiplicities and the number of liver tumors less than 2 mm^2 in size. Moreover, BCA could inhibit DNA synthesis in adult mice. However, BCA is also defined as a phytoestrogen compound in the isoflavone family. Given that the administration of testosterone, an androgen, to female mice would have a differentiated impact on the number of liver tumors, further analyzing whether the estrogenic activity of BCA itself has a negative regulatory effect on hepatocarcinogenesis may be beneficial to clarify gender-related liver tumor induction, that is, whether sexual dimorphism exists in hepatocarcinogenesis[78].

3.6. Inhibiting CYP24A1 hydroxylase

Vitamin D is a fat-soluble seco-steroid with potential anticancer activity. However, its current anticancer efficacy in the clinic is limited because its bioactive form, calcitriol, can be inactivated under the mediation of the highly active cytochrome P450 family 24 subfamily A member 1 (CYP24A1) hydroxylase in cancer cells[80,81]. Therefore, in targeted HCC treatment, down-regulating the activity of CYP24A1 hydroxylase in HCC cells may extend the half-life of calcitriol in the body by 10 times, thereby maintaining the effectiveness of vitamin D's metabolically activated form[81]. Normoxia (21% O_2) and hypoxia (1% O_2) environments can induce CYP24A1 overexpression by calcitriol. However, hypoxia mimics the microenvironment of solid tumors and triggers treatment resistance. Therefore, hypoxia stimulation remarkably induces CYP24A1[81]. BCA could not only reverse overexpressed CYP24A1 in the HCC cell line HuH-7 induced by calcitriol in a normoxic environment to the control level, but also regulate CYP24A1 expression to approximately four times that of the control group under hypoxia signal stimulation.

Therefore, ingesting the isoflavone compound BCA from red clover extract or legumes and nuts is beneficial to anti-CYP24A1 gene transcription and strengthens the antitumor activity of vitamin D[81] (**Fig. 2**).

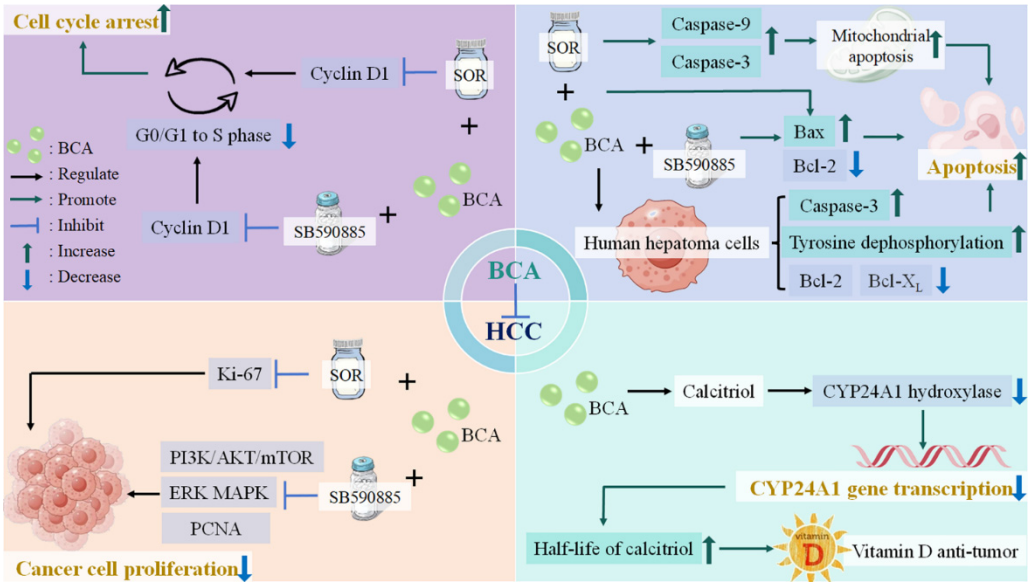

Fig. 2. Anti-HCC mechanisms of BCA. The combined treatment of BCA with SOR or SB590885 could inhibit the expression of cyclin D1, hindering cell cycle transition from the G0/G1 phase to the S phase, that is, inducing HCC cell cycle arrest. The combined treatment of BCA with SOR could promote the overexpression of caspase-3 and caspase-9, thereby inducing mitochondrial apoptosis. In addition, the combined treatment of BCA with SOR or SB590885 could up-regulate the expression of Bax/Bcl-2, further leading to the apoptosis of HCC cells. In human hepatoma cells, BCA could induce the tyrosine dephosphorylation of cellular proteins and the activity of caspase-3, as well as down-regulate the expression of Bcl-2 and Bcl-X_L. These effects ultimately promoted the apoptosis of human hepatoma cells. Cotreatment with BCA and SOR could inhibit the expression of Ki-67, whereas synergistic treatment with BCA and SB590885 could down-regulate the ERK MAPK and PI3K/AKT/mTOR pathways and reverse PCNA overexpression, thereby suppressing the proliferation of HCC cells. In HCC cells, BCA could inhibit the calcitriol-induced overexpression of CYP24A1 hydroxylase, thereby reducing CYP24A1 gene transcription and prolonging the half-life of calcitriol, the bioactive form of vitamin D. Ultimately, these mechanisms achieve vitamin D anti-HCC therapy.

4. The protective mechanisms of BCA against MASLD

4.1. Metabolic dysfunction-associated steatotic liver disease

MASLD is the most widespread phenotype of chronic liver diseases in the Asia-Pacific region. Its prevalence is increasing rapidly, and it has affected more than one quarter of the global population[82,83]. In addition, it is the second leading cause of liver transplantation in the United States and Europe, and its overall global prevalence is estimated to be 32.4%[4]. MASLD, a chronic and heterogeneous disease, is manifested by the production of lipid droplets (LDs) in more than 5% of hepatocytes. Imbalanced LD biogenesis and degradation further induce intracellular lipid deposition and steatosis and finally drive the occurrence of hepatocyte inflammation infiltration and fibrosis[84]. Therefore, the inappropriate prognosis of MASLD often exacerbates the development of its advanced subtypes, such as metabolic dysfunction–associated steatohepatitis (MASH), which is the result of liver injury deterioration and can further induce liver cirrhosis and HCC[85].

The two-hit hypothesis still explains the lipotoxic mechanisms of MASLD. The development of MASLD is generally believed to be regulated by dual factors. First, triglycerides (TG) deposited in hepatocytes drive the occurrence of hepatic steatosis. Second, the secretion of proinflammatory cytokines and enhancement of oxidative stress induce liver fibrosis[86]. However, the multiple-hit hypothesis, which

involves multiple effects, such as insulin resistance (IR), damaged adipocyte function, gut microbiota imbalance, immune regulation disorders, and dietary habits, is an optimization of the two-hit hypothesis, which is based only on lipid metabolism imbalance and inflammatory factors[87]. Notably, the core elements of the two- and multiple-hit hypotheses both point to lipid metabolism imbalance[88]. Considering that multiple in vivo and in vitro studies have demonstrated the potential of BCA as a regulator of glycolipid metabolism and an agonist of farnesoid X receptor (FXR) and autophagy, BCA may be an effective bioactive substance for targeting MASLD.

4.2. Regulating glycolipid metabolism

4.2.1. Glucose metabolism

As a cornerstone of MASLD, IR can further promote gluconeogenesis, glycolysis, and *de novo* lipogenesis, thereby inducing hepatic glucose output and triggering chronic glucotoxicity–mediated liver inflammation and fibrosis[89]. Hee-Sook Park et al. reported that the expression levels of the hepatic glucose metabolism intermediates fructose 6-phosphate and dihydroxyacetone phosphate and the PPARα target glucose-6-phosphatase (G6Pase) were significantly down-regulated in high-fat diet (HFD) mice under BCA intervention. That is, BCA could inhibit glucose-6-phosphate-mediated gluconeogenesis. In addition, in HFD mice, pyruvate, the main precursor of gluconeogenesis, was heavily converted into lactic acid, and supplementation with BCA could further down-regulate lactic acid levels. In HFD mice, BCA also effectively inhibited pyruvate kinase, which is the final rate-limiting enzyme of the aerobic glycolysis pathway, and the overexpression of glucose transporter 2 in the liver[90]. Furthermore, the hepatic transcription factor carbohydrate responsive element binding protein (ChREBP) plays an important role in mediating hepatic lipid synthesis, the glucose-induced glycolytic enzyme L-pyruvate kinase, and acetyl-coenzyme A carboxylase (ACC). However, BCA supplementation could reverse the activation of ChREBP in the liver of HFD-treated mice. In conclusion, BCA supplementation was beneficial for alleviating the imbalance of gluconeogenesis and glycolysis reactions in diet-induced obesity (DIO) mice[90]. Notably, the mechanism by which BCA reduced gluconeogenesis in HFD-induced mice did not involve considerable changes in the gene expression levels of insulin receptors and insulin receptor substrate 1[90]. Not only that, BCA also reversed the HFD-induced up-regulation of fasting blood glucose levels, serum insulin content, glucose tolerance, and IR index and enhanced insulin sensitivity, thereby effectively alleviating the HFD-induced disorder of glucose metabolism and MASLD[88,90]. Finally, glucose tolerance tests conducted at 7 weeks showed that in DIO mice, BCA supplementation promoted the immediate clearance of blood glucose levels. That is, BCA induced the recovery of blood glucose parameters and insulin sensitivity in DIO mice[90].

4.2.2. Lipid metabolism

Obesity is a triggering factor of metabolic syndromes, such as hyperlipidemia and IR, and obesity-related visceral lipid deposition may induce hepatic dysfunction[91,92]. Considering the effectiveness of natural plant-derived compounds and preparations containing plants in alleviating obesity

symptoms, relevant studies have confirmed the potential of BCA for improving metabolic disorder–related lesions, such as hepatic steatosis, in DIO mice[88,90,93]. BCA intervention could significantly down-regulate the Lee index and liver index, which are similar to the human body mass index. That is, in rats, BCA could relieve the induction of visceral obesity by HFD but had no significant effect on weight. Furthermore, although HFD up-regulated cholesterol synthesis, BCA further promoted the bioconversion of cholesterol into bile acid and the excretion of cholesterol in feces by inducing cholesterol 7α-hydroxylase (CYP7A1), a rate-limiting enzyme in bile acid synthesis. BCA reduced the overexpression of sterol regulatory element binding protein-1c (SREBP-1c) and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) in the liver to inhibit cholesterol synthesis. At the same time, BCA drove the enhanced activity of PPARα and low-density lipoprotein receptor (LDLR) in the liver and down-regulated the level of PPARγ, further strengthening cholesterol absorption and metabolism[88]. Therefore, in the end, BCA reversed the HFD-induced increase in total cholesterol (TC), TG, and LDL and increased high-density lipoprotein levels, thereby effectively alleviating HFD-induced lipid metabolism disorders and MASLD[88,90]. Notably, in an in vitro model based on the human HCC cell line HepG2, BCA also promoted the activity of LDLR on the cell surface, deriving the enhanced hepatic clearance of LDL cholesterol from plasma[94]. It is generally believed that natural products rich in estrogen act as LDLR regulators mainly because of the influence of estradiol. However, the phenolic hydroxyl group in the chemical structure of 17β-estradiol can not only form hydrogen bond interactions with the amino acid structures present in the ER, but also determine the potential of 17β-estradiol as an antioxidant. Considering that antioxidants possess the bioactivity of LDLR induction, whether the overexpression of LDLRs in HepG2 cells driven by phytoestrogen compounds is due to their own estrogenic effects or the antioxidant activity derived from them remains ambiguous[94]. Similar findings were also observed by Xue et al., who discovered that BCA could induce hepatic triglyceride lipase (HTGL) activity to enhance cholesterol uptake in the liver and further inhibit TG deposition in the livers of HFD mice by up-regulating hepatic lipoprotein lipase (LPL) expression. Secondly, BCA could promote the up-regulation of serum and hepatic SOD activities in HFD mice while inhibiting MDA and reactive oxygen species (ROS) levels, thereby effectively alleviating HFD-induced lipid peroxidation (LPO) damage to liver function on the basis of its free radical scavenging property[95]. In addition, HFD-induced mice exhibited swollen hepatic morphology, and BCA intervention had significant improvement benefits on the increase in the activities of serum hepatic function indicators AST and ALT and hepatic steatosis and inflammatory cell infiltration driven by HFD[88,90]. On the other hand, BCA intervention promoted the overexpression of PPARα and its target proteins peroxisome proliferator–activated receptor gamma coactivator 1 alpha (PGC–1α), carnitine palmitoyltransferase 1 alpha (CPT-1α), uncoupling protein 2 (UCP2), and acyl-coenzyme A oxidase 1 (ACOX1) in the livers of HFD-treated mice and induced the inactivation of ACC and fatty acid synthase (FAS), the rate-limiting enzymes of lipid synthesis. That is, BCA might enhance the driving effect of PPARα on mitochondrial fatty acid (FA) β-oxidation and mediate the inhibition of lipid synthesis, thereby triggering hepatic fat-reducing signals[90]. Furthermore, in

HFD-treated mice, BCA significantly promoted the up-regulation of the FA transporters carnitine and acetylcarnitine content by 1.2 and 7.7 times, respectively. It also promoted the phosphorylation of adenosine monophosphate–activated protein kinase (AMPK)[90]. Not only that, the production of hepatic ketone bodies, such as β-hydroxybutyric acid (β-HB) and acetoacetate, depends on the oxidation of hepatic FAs as a synthetic fuel. The content of β-HB in the livers of mice supplemented with BCA was 1.4 times higher than that in mice treated with HFD alone. Finally, BCA potentially targeted the enhanced mitochondrial oxygen consumption rate of palmitic acid–induced HepG2 cells to mobilize oxidative capacity. This finding indicated that BCA might induce the oxidation of FAs by promoting the synthesis of ketone bodies and transportation of certain necessary components into mitochondria[90].

The *de novo* phosphatidylcholine or cytidine diphosphate–choline (citicoline) synthesis pathway mediates approximately 70% of the synthesis of hepatic phosphatidylcholine. Phosphatidylcholine plays an important role in the packaging and export of TG in the liver, and citicoline and diacyl glycerol (DAG) are important substrates in the choline/ethanolamine phosphotransferase 1 (CEPT1)–mediated *de novo* synthesis of phosphatidylcholine[96]. Among them, DAG synthesized from the derivative glycerol-3-phosphate can be mobilized for the storage of acylglycerols or induce phosphatidylcholine synthesis[97]. In HFD mice, supplementation with BCA further drove phosphatidylcholine synthesis by up-regulating the content of citicoline and DAG in the liver and overexpressing CEPT1. In the final stage of TG biosynthesis in the liver, DAG, a lipid component composed of a glycerol molecule linked to two FAs through ester bonds, is acylated by diacylglycerol acyltransferase 1 (DGAT1) to generate TG. The livers of HFD-treated mice showed remarkable steatosis and DGAT1 overexpression. Notably, BCA supplementation only inhibited TG accumulation in the livers of HFD mice but did not affect DGAT1 expression. This effect indicated that BCA treatment activated the metabolites and genes related to the phosphatidylcholine synthesis pathway and drove the *de novo* synthesis of phosphatidylcholine in the livers of DIO mice while inducing the inhibition of lipogenesis[90]. Furthermore, choline, as an essential nutrient that is closely related to MASLD, affects various life phenomena, such as lipid metabolism and lipid second messenger–mediated signal transduction. The liver is the main biological site for the oxidation of choline into betaine. This oxidation process can be divided into two major pathways: mitochondrial choline oxidase first drives the biotransformation of choline into betaine aldehyde, and betaine aldehyde is then further oxidized and induced into betaine[98]. However, BCA supplementation up-regulated the content of betaine aldehyde and betaine in the liver. That is, BCA might restore the homeostasis of hepatic lipid metabolism by activating choline catabolism in HFD mice. In conclusion, BCA further alleviates hepatic steatosis by targeting lipid oxidation, *de novo* lipid synthesis, cholesterol absorption and excretion, phosphatidylcholine synthesis, and choline metabolism[90].

4.3. Promoting autophagy

MASLD is often accompanied with the deposition of damaged organelles, such as LDs, mitochondria, endoplasmic reticulum, and peroxisomes, in cells. Therefore, activating autophagy degradation to clear

damaged structural components, that is, targeting the autophagy pathway, has gradually been defined as an effective regulatory strategy for alleviating MASLD[99]. AMPK and mitochondrion-localized sirtuin 3 (SIRT3) are upstream effectors of the autophagy-initiating molecule Unc-51-like kinase 1 (ULK-1)[100]. Adipocyte differentiation–related protein (ADRP) on the surfaces of hepatic LDs is closely related to the regulation of lipid accumulation and activation of the hepatic lipid overload response. Therefore, its ablation may drive lipophagy to degrade the content of supersaturated LDs through the induction of sequestosome 1 (p62)– and microtubule-associated protein 1 light chain 3 (LC3)–mediated autophagic vesicles[101]. This phenomenon suggests that the autophagy-promoting effect is beneficial for relieving hepatic steatosis. The chickpea-derived isoflavone compound BCA could target the up-regulation of SIRT3 protein expression and AMPK phosphorylation, among which SIRT3 was also an upstream signaling molecule that activated AMPK. In HepG2 cells induced by oleate (OA), BCA enhanced phosphorylated AMPK activity, further promoted ULK-1 phosphorylation, and finally drove the overexpression of the autophagosome formation biomarkers Beclin 1 and LC3-II and inhibited the accumulation of the autophagic flux biomarker p62, thus mobilizing ULK-1-mediated lipophagy. It thus further promoted ADRP inactivation in steatotic hepatocytes, thereby leading to the attenuation of intracellular TG synthesis and alleviating steatosis[100,102]. Furthermore, phosphorylated AMPK can also further drive the phosphorylation of ACC. Therefore, it is considered as a prerequisite for inactivating ACC and inhibiting hepatic FA overload[103]. In summary, BCA alleviated lipid deposition in OA-induced HepG2 cells through the activation of the SIRT3/AMPK/ULK-1 signaling cascade in steatotic hepatocytes and its induced autophagy effect[100].

4.4. Activating FXR and inhibiting inflammation

The ligand-activated transcription factor FXR is highly expressed in the liver and effective in regulating lipid and bile acid metabolism. Therefore, the FXR agonist obeticholic acid has been approved by the FDA as a second drug for the treatment of primary biliary cholangitis and can also be utilized in phase III clinical trials for the treatment of MASH[104,105]. Although obeticholic acid has achieved the endpoint of fibrosis improvement to some extent, its potential adverse effects, such as pruritus and cholelithiasis, have limited its regulatory approval as a therapeutic agent for MASH[104,106]. Wang et al., who aimed to obtain FXR agonists with moderate benefits far exceeding their safety risks by identifying several natural product–derived FXR agonists, discovered that most of the screened FXR agonists were members of the isoflavone class of compounds. Moreover, structure–activity relationship analysis based on chemical skeletons demonstrated that the localization of the B ring at the C3 position determined FXR activation bioactivity[107]. FXR is a pleiotropic anti-inflammatory effector molecule that acts by inhibiting the transcription of the NF-κB p65 subunit and proinflammatory cytokines[108]. Therefore, the ablation of the FXR protein is inconducive to leveraging the hepatoprotective effect of FXR agonists fully. However, among identified natural active products, BCA not only directly bound to and transactivated FXR, its anti-inflammatory effect was not dependent on activating FXR. This characteristic was mainly demonstrated by the fact that in hepatocytes transfected with *Fxr* small interfering RNA (siRNA), BCA could still

markedly inhibit the mRNA overexpression of tumor necrosis factor-α (TNF-α)[107]. Notably, further verification through in vivo animal experiments is needed to determine whether the anti-inflammatory property of BCA, which is independent of FXR, exerts hepatoprotective benefits in MASH models[107]. Finally, BCA also markedly improved lipid deposition in palmitic acid–Na-induced HepG2 cells, further highlighting the potential of this natural FXR agonist as a lead compound of plant origin for the dual targeting of lipid metabolism imbalance and inflammation in MASH treatment (**Fig. 3**).

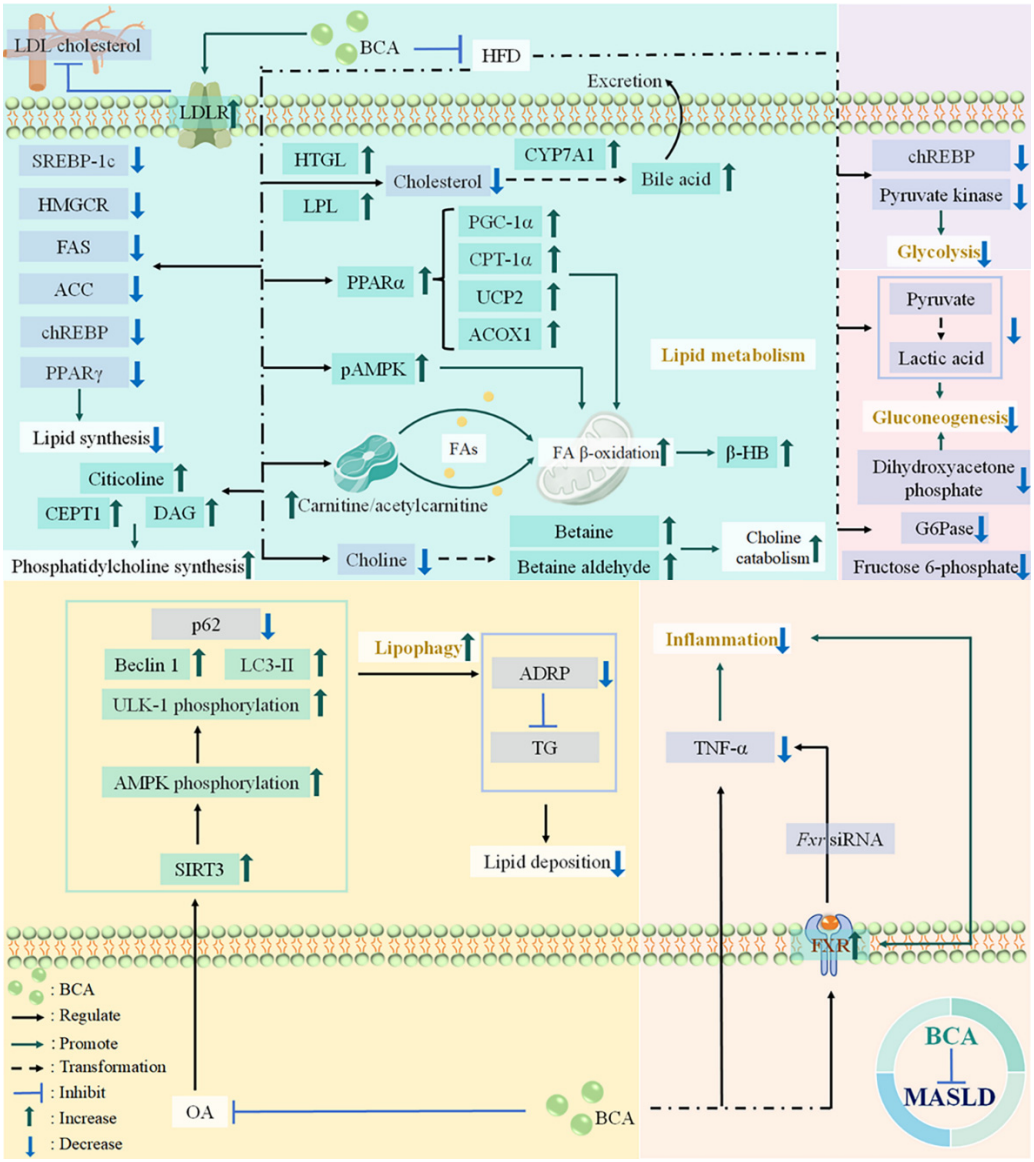

Fig. 3. Anti-MASLD mechanisms of BCA. BCA interfered with lipid synthesis by inhibiting the HFD-induced overexpression of SREBP-1c, HMGCR, FAS, ACC, chREBP, and PPARγ. BCA stimulated the β-oxidation of FAs in mitochondria by promoting the expression of PPARα and its target proteins, enhancing the phosphorylation of AMPK, and inducing the transport of FAs by carnitine and acetylcarnitine, which also provided fuel for the synthesis of β-HB. BCA up-regulated the activities of HTGL and LPL to alleviate hepatic lipid deposition. BCA induced the biotransformation of cholesterol into bile acids and the excretion of cholesterol by enhancing the expression of CYP7A1. BCA drove the *de novo* synthesis of phosphatidylcholine by inducing citicoline, DAG, and CEPT1. BCA promoted the catabolism of choline, thereby up-regulating the levels of betaine and betaine aldehyde. The induction effect of BCA on hepatic LDLRs promoted the clearance of LDL cholesterol in plasma. BCA inhibited glycolysis by down-regulating the activities of chREBP and pyruvate kinase, potentially reduced the conversion of pyruvate into lactic acid, and suppressed the expression of hepatic fructose 6-phosphate, dihydroxyacetone phosphate, and G6Pase, thereby alleviating overactivated gluconeogenesis. BCA activated the SIRT3/AMPK/ULK-1 pathway to trigger lipophagy, promoting ADRP inactivation and attenuating hepatic TG deposition. BCA exhibited anti-inflammatory effects independent of hepatic FXR activation, indicating it could still inhibit the release of TNF-α in hepatocytes transfected with *Fxr* siRNA.

5. The protective mechanisms of BCA against liver fibrosis

5.1. Liver fibrosis

Chronic liver disease is an immeasurable threat to global public health, with approximately 30% of patients with this condition being susceptible to fibrosis and cirrhosis[26]. Although hepatocytes have the potential for compensatory replication, liver regenerative ability is impaired, which will considerably shorten survival, when fibrosis deteriorates into decompensated cirrhosis[109]. Chronic progressive liver fibrosis with an inappropriate prognosis is characterized by excessive hepatic scar tissue hyperplasia and often ends with liver cirrhosis. It has become a major threat to the health of the more than 600,000 individuals in the adult population of the United States[86]. In addition, because liver cirrhosis causes approximately more than 1 million deaths each year, it ranks 11th among the most common causes of death worldwide. It is also a potential pathological factor that affects the health of 1%–2% of the global population[110]. At present, HCV infection, alcoholic liver disease (ALD), MASLD, and drug-induced liver injury (DILI) constitute the external causes of liver fibrosis[111]. Apoptosis, oxidative stress, inflammation, epigenetics, and receptor-mediated signaling are the endogenous factors involved in liver fibrosis. Exploring the profibrotic microenvironment in liver tissue and regulatory mechanisms of immune cells and macrophages has also gradually become a hotspot in the research on liver fibrosis[6]. Liver fibrosis can be attributed to excessive extracellular matrix (ECM) deposition, which becomes a potential cause of cell stress, tissue damage, organ dysfunction, and even death[112]. In short, various factors indicate that developing new and highly effective antifibrotic drugs and avoiding the biotoxicity associated with chronic drug exposure as much as possible are urgently needed. Natural products have gradually attracted attention because of their rich reserves, wide biological sources, and lower risks compared with synthetic drugs[113]. This review focuses on the anti-hepatic fibrosis potential of BCA, which has been demonstrated to exert remarkable pharmacological benefits in reversing liver fibrosis by inhibiting the secretion of cytokines and proliferation of activated hepatic stellate cells (HSCs) or depending on bioactivities, such as antifree radical and anti-inflammatory cell infiltration activities.

5.2. Inhibiting inflammation and oxidative stress

5.2.1. Inflammation

After carbon tetrachloride (CCl_4) damages the liver parenchyma, NF-κB is markedly activated in Kupffer cells and then drives the secretion of the inflammatory cytokine TNF-α and fibrotic marker molecule transforming growth factor-β1 (TGF-β1). Among them, TGF-β1, as a growth factor that regulates fibrotic effects in multiple dimensions, can induce the phenotypic transformation of hepatocytes into myofibroblast-like cells and the synthesis and deposition of ECM components[114,115]. Considering the effectiveness of BCA in inhibiting the expression of the proinflammatory cytokine TNF-α and profibrotic effector TGF-β1 and its biological characteristics as a NF-κB signal regulator, BCA may be a potential natural anti-fibrosis therapeutic agent that acts through mediating intercellular communication[116]. Furthermore, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) can act as biological

enzymes that initiate inflammatory cascades and participate in inflammatory signal transduction, with the latter enhancing the synthesis of prostaglandins under the mediation of an inflammatory environment[117]. The bioactivity of BCA in inhibiting liver fibrosis could also be attributed to its anti-inflammatory effect, which is achieved by targeting the down-regulation of iNOS and COX-2 activities[116].

5.2.2. Oxidative stress

Cytochrome P450 2E1 (CYP2E1), a member of the CYP450 family of phase I metabolizing enzymes, is involved in driving the biotransformation of exogenous toxic compounds, such as CCl_4, in the liver[118]. However, BCA could target the decrease in CYP2E1 activity and even regulate its expression to be lower than that in controls[116]. In addition, CYP1A1 is another classical P450 enzyme that drives oxidative stress marked by excessive ROS release, which can further lead to the progressive aggravation of liver injury. Notably, BCA not only showed the biological benefit of inducing its inactivation but enhanced the up-regulation of the activity of the phase II enzyme sulfotransferase 1A1 (SULT1A1) that synergistically promoted the metabolic clearance of exogenous substances from the liver[116,119]. Furthermore, the derivation of free radicals to induce oxidative stress is the primary mechanism by which CCl_4 triggers cytotoxicity. Oxidative stress further promotes the activation of diverse kinases that mediate the NF-κB pathway, and the excessive release of ROS can also induce the DNA binding activity of NF-κB. These pathological factors may be closely related to the occurrence and progression of fibrosis. However, BCA pretreatment could regulate MDA expression and total antioxidant capacity (TAC) recovery and alleviate the sharp down-regulation of GSH[116,120,121].

Thioacetamide (TAA), although it can be used as an organic sulfur fungicide in the textile industry, is also often employed to induce hepatotoxicity and has been identified as a compound with carcinogenic effects[122,123]. In addition, TAA can be used as an inducer to simulate human fibrosis in experimental animals. Although this chemical induction is almost irreversible, the administration of 25 mg/kg or 50 mg/kg BCA after TAA treatment significantly reduced the liver weight of rats and cell damage[26]. TAA mainly induces hepatotoxicity and fibrosis by driving the release of free radicals and triggering oxidative stress. In liver homogenates, these effects manifested as the inactivation of antioxidant enzymes, such as SOD, CAT, and glutathione peroxidase (GPx), and the up-regulation of MDA levels. However, amphiphilic flavonoid compounds effectively inhibit radical chain reactions by trapping chain-initiating radicals at the cell membrane interface. Moreover, these substances can provide hydrogen atoms from their aromatic hydroxyl groups to free radicals, further forming stable phenolic radical structures and thereby exhibiting remarkable antioxidant activity. As a representative amphiphilic flavonoid compound, BCA could effectively reverse the down-regulation of the above endogenous antioxidant enzymes and the overexpression of the LPO indicator MDA, thereby resisting free radical damage to hepatocytes[26].

5.3. Inhibiting HSC activation/proliferation and hepatocyte propagation

The activation of HSC is closely related to the proliferation of cells, deposition of ECM, and the overexpression of alpha-smooth muscle actin (α-SMA) in myofibroblasts[124]. TAA promotes the release of

ROS, thereby initiating the proliferation of HSCs, further inducing the synthesis and deposition of ECM associated with chronic liver cirrhosis and activating TGF-β1 and α-SMA[26]. In addition, in view of the regenerative and repair capacity of the liver, TAA hepatotoxicity also indirectly leads to the compensatory proliferation of hepatocytes. During the S phase of mitosis, the expression of PCNA, the auxiliary protein of DNA polymerase, is often enhanced[26,125]. However, in contrast to TAA-induced liver cirrhosis, additional BCA intervention could effectively inhibit the α-SMA, PCNA, and mitotic index, thereby down-regulating the excessive propagation of hepatocytes attempting to repair liver injury. Furthermore, BCA markedly alleviated the driving effect of excessive ROS release on HSC activation. The up-regulation of the TAA-induced serum-specific liver enzymes alkaline phosphatase (ALP), ALT, AST, and gamma-glutamyl transferase (GGT) considerably reversed after BCA treatment[26]. Notably, the overexpression of matrix metalloproteinases (MMPs) drive the enhanced degradation of normal subendothelial ECM and accelerate the replacement of its physiological structure by over-deposited collagen fibers, ultimately forming a pro-fibrotic positive feedback loop through inducing the activation of HSCs[126]. Among them, the CCl_4-targeted up-regulation of MMP-9 is a potential factor that may exacerbate hepatotoxicity. Moreover, the activation of the liver fibrosis marker MMP-9 is also significantly positively correlated with the overexpression of TNF-α and TGF-β1. However, these phenomena could all be reversed by BCA[116,127]. Finally, BCA had the pharmacological property of inhibiting the expression of α-SMA, a marker of HSC activation induced by CCl_4; this property was also a mechanism through which it exerted its anti-hepatic fibrosis effect[116]. Moreover, platelet-derived growth factor (PDGF) is an inducing factor that drives the activation and proliferation of HSC and accounts for 50%–70% of the total macrophage-derived mitogenic activity[128]. Considering that flavonoids have been proven to be a class of potential phytoestrogens and hepatoprotective agents with characteristics of anti-radical generation and anti-oxidative stress[129] and inhibition of tyrosinase activity[130] and apoptosis[131], some bioactive substances from natural resources have been confirmed to reverse the inductive effect of PDGF on the proliferation of rat HSCs. Among them, five flavonoids including BCA had an inhibitory activity of more than 75% against PDGF-derived HSC-T6 proliferation induction effect, which was closely related to the ubiquity of the 4′-hydroxy group in their chemical structures[128].

5.4. Inhibiting schistosome infection

Schistosomiasis, one of the most common parasitic fibrotic diseases, can cause the deposition of a large amount of scar tissue around ova trapped in the liver and hepatic inflammation infiltration[132]. Although some *Schistosoma* eggs can be excreted from the body through the fecal route, some may be deposited in the liver. *Schistosoma* eggs trapped in the liver tissue for a long time can trigger chronic inflammation, further induce the formation of granulomas, and may even promote the abnormal proliferation of HSCs and the excessive deposition of ECM. Eventually, fibroblasts in the liver gradually transdifferentiate into myofibroblasts; that is, liver fibrosis occurs[132]. Although praziquantel is listed by the World Health Organization as the only recommended drug for the prevention and treatment of schistosomiasis, the use of a

single drug for the targeted therapy of this disease may eventually lead to drug resistance in schistosomes. Moreover, the effectiveness of praziquantel only applies to adult worms and has no considerable effects on schistosomula and pre-adult worms or juvenile worms[132–134]. Therefore, promoting the development of anti-schistosomal agents with considerable therapeutic benefits and low risks is of great value. Schistosomiasis plagues approximately 250 million people from 78 countries and causes the deaths of approximately 280,000–500,000 people each year. In view of the threat of schistosomiasis to health undertakings, scientists have begun to pay attention to the feasibility of applying bioactive agents derived from natural products to intervene in this disease[135,136]. Research showed that a single-dose BCA intervention could significantly down-regulate worm burden in the early and late stages of infection, as well as mean tissue egg load, and inhibit the expansion of granulomas[132]. In addition, the expression of schistosome CYP450 is a key factor determining worm survival rate and egg development. At present, anti-schistosomiasis treatment measures targeting CYP450 have been approved for verification in mice, and CYP450 inhibitors at micromolar to nanomolar concentrations have all demonstrated certain anti-worm benefits[137–139]. Notably, BCA could also act as a potential CYP450 mRNA inhibitor to exert pharmacological effects. BCA intervention in the early stage of schistosome infection often had a more significant inhibitory effect on CYP450 mRNA than that in the later stage. That is, BCA treatment may be needed during the early infection period to relieve total worm burden and kill immature parasites effectively[132]. The transformation of activated HSC into myofibroblasts and deposition of ECM proteins around eggs are involved in the occurrence of *Schistosoma* liver fibrosis. Among them, the fibrotic effector molecule TGF-β is an inducing factor for ECM synthesis and deposition and mesenchymal cell proliferation, migration, and accumulation after inflammatory infiltration[132]. In addition, COX-2 is not only a rate-limiting enzyme in the synthesis of the inflammatory mediator prostaglandin E2. COX-2 positive cells can also serve as an important source in the area of highly activated fibrotic front and mobilize the pro-fibrotic properties of fibroblastic foci. Therefore, COX-2 overexpression can further drive the occurrence of liver fibrosis by mediating proinflammatory effects and inducing fibroblast transdifferentiation[132,140]. Not only that, the proinflammatory effects driven by schistosome infection also exacerbate the imbalance of antioxidant responses in the host. This effect may be accompanied with the excessive generation of pro-oxidative stress effectors, such as nitric oxide (NO) and iNOS. However, BCA administered at the early or late stage of infection can inhibit the overexpression of TGF-β, COX-2, and iNOS, indicating its effectiveness in the targeted treatment of schistosomiasis[132] (**Fig. 4**).

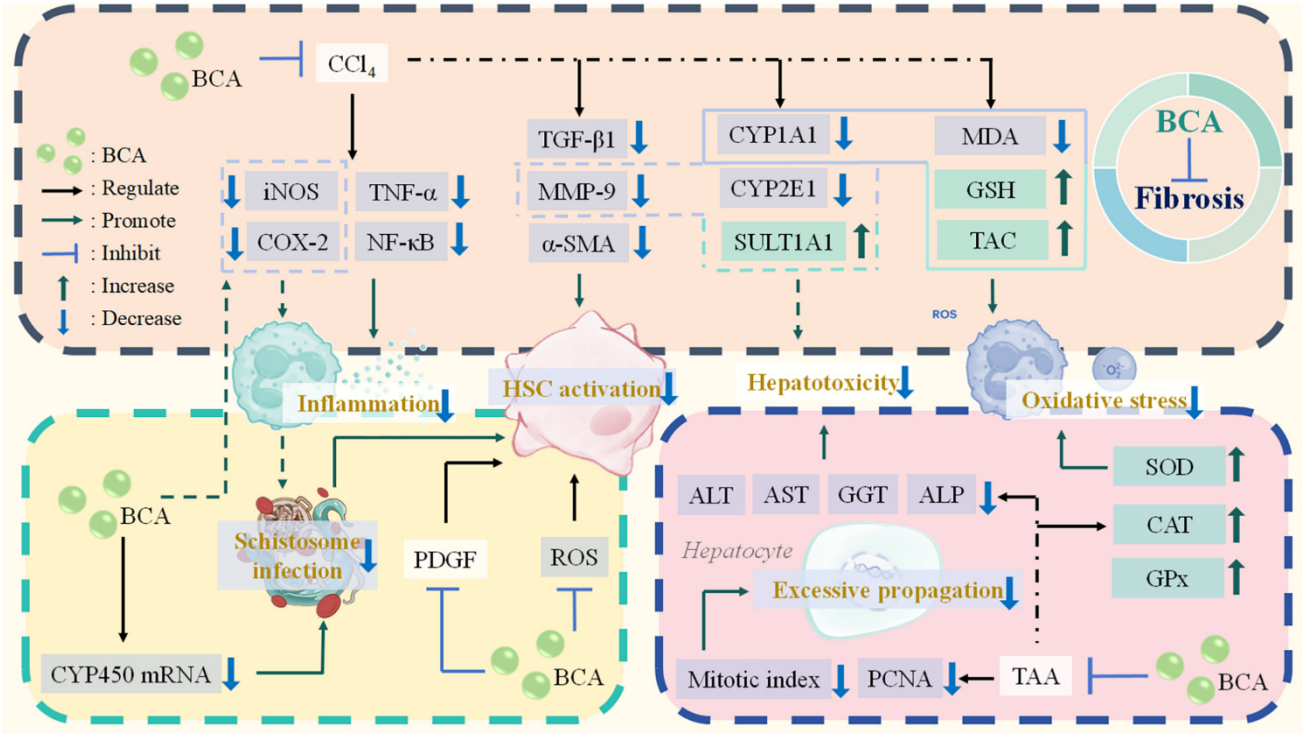

Fig. 4. Mechanisms of BCA in anti-liver fibrosis. BCA could alleviate hepatic inflammatory infiltration by inhibiting the CCl_4-induced overexpression of COX-2, TNF-α, NF-κB, and iNOS and could reverse the driving effects of MMP-9, TGF-β1, α-SMA, ROS, and PDGF on HSC activation. Additionally, BCA could up-regulate SULT1A1 expression and down-regulate CYP2E1 and CYP1A1 activities. This regulatory effect not only mitigated CCl_4-induced hepatotoxicity but allowed the inactivated CYP1A1, alongside BCA-induced antioxidants, to potentially combat oxidative stress. Furthermore, BCA could inhibit TAA-induced serum liver enzyme levels and further alleviate the excessive propagation of hepatocytes attempting to repair damage by down-regulating PCNA expression and the mitotic index. Not only that, the anti-inflammatory and anti-oxidative stress effects of BCA exerted through the inhibition of COX-2 and iNOS, as well as its anti-worm effect induced by the targeted down-regulation of CYP450 mRNA expression, would mitigate the HSC activation driven by schistosome infection and its contribution to liver fibrosis.

6. The protective mechanisms of BCA against DILI/hepatotoxicity

6.1. Drug-induced liver injury/hepatotoxicity

Almost all nutrients absorbed in the small intestine need to be transported to the liver first through the hepatic vein and processed by the liver before they can be transported to various tissues in the body. Therefore, the liver acts as the metabolic hub of various exogenous substances in the digestive system. The xenobiotics recognized in the liver, including alcohol, heavy metals, drugs, and chemicals, may attack the liver parenchyma and trigger DILI or hepatotoxicity[141,142]. Among them, the toxic effect of heavy metals is mainly mediated by triggering the oxidative damage of released ROS to various organs. The liver, as the main target organ attacked by heavy metals, often experiences the complications of cholestasis, lipid deposition, vascular lesions, and liver necrosis. In addition, heavy metal stimulation can activate caspases, induce hepatocyte apoptosis, liver ultrastructure damage, and epigenetic lesions[143]. For decades, metal chelators have been considered as the first choice for the treatment of heavy metal poisoning. However, chelation therapy exhibits poor specificity and requires cumbersome administration. Moreover, it can indirectly damage soft tissue structures, such as the liver. Therefore, its potential side effects limit its long-term feasibility[144]. DILI is usually classified as predictive, idiosyncratic, and indirect. Predictive DILI has a direct dose-dependent relationship. The risk of its occurrence is related to drug exposure and can

be reproduced in animal models. Therefore, this type of liver injury is predictable[145]. Although idiosyncratic liver injury does not directly depend on dose, drugs with oral daily dose ≥ 50 mg are often the most common causes of liver injury[146]. While DILI has a low incidence rate, its phenotypes include almost most types of liver injury. In accordance with the degree of drug exposure, DILI can clinically manifest as the nonspecific elevation of hepatic biochemical indicators in mild cases and as jaundice, fulminant liver failure, and even ultimately death in severe cases[147]. DILI often resolves spontaneously with the discontinuation of the suspected drug. However, in autoimmune-like DILI, further intervention with corticosteroid hormones is often required[146]. Notably, DILI with poor prognosis may deteriorate into acute liver failure, and liver replacement therapies, such as liver transplantation, cannot extend the survival of some patients with lethal acute liver failure[148]. Therefore, considering that natural products are more cost-effective and have higher bioactivity and lower medical risk than most prescription drugs for liver diseases, they are gradually being favored as a complementary therapy for alleviating DILI/hepatotoxicity[149].

6.2. Ritonavir treatment-induced DILI

The long-term exposure of the liver to high concentrations of exogenous drugs or non-infectious pathogens may induce susceptibility to hepatotoxicity. Therefore, the liver is generally defined as the main target organ of drug-induced injury[150,151]. Although ritonavir (RIT) can be used as a human immunodeficiency virus protease inhibitor, it is often accompanied with dose-dependent hepatotoxicity. At present, the caspase cascade system is believed to be involved in the induction of RIT-related hepatotoxicity[150,152]. However, red clover isoflavones have anti-inflammatory, antioxidant and anti-apoptotic activities. BCA, as one of the bioactive components, can inhibit the overexpression of Bax, caspase-3, caspase-8, NF-κB, and endothelial nitric oxide synthase (eNOS) induced by RIT, and preventive treatment with BCA has been found to maintain the activity of the anti-apoptotic gene Bcl-2[150]. In addition, the contents of serum glutamatic oxaloacetic transaminase (SGOT), serum glutamic pyruvate transaminase (SGPT), and ALP are often defined as quantitative indicators of the degree of liver injury. However, BCA not only negatively regulated the content of serum enzymes induced by RIT, it down-regulated the levels of nitrite and the LPO indicator MDA induced by RIT. It also increased the content of the antioxidant GSH. In this process, the reduction in nitrite content accordingly inhibited the release of NO. Furthermore, after BCA intervention, the RIT-induced overexpression of the inflammatory enzymes COX and lysyl oxidase (LOX) in serum and the release of the proinflammatory factors interleukin-1β (IL-1β) and IL-6 were down-regulated, whereas the anti-inflammatory factor IL-10 was up-regulated. Not only that, BCA pretreatment could up-regulate the expression of the pro-survival factor p-AKT and inhibit the induction of hepatocyte death by adverse stress. In short, by targeting oxidative stress, inflammation, and apoptosis and reversing the mild focal mononuclear cell infiltration in liver tissue induced by RIT, BCA acts as a natural anti-hepatotoxic immunomodulator that can be applied in the complementary therapy of antiretrovirals[150].

6.3. Bosentan treatment-induced DILI

Organic anion transporting polypeptide 1B1 (OATP1B1), a specific uptake transporter that is mainly located on the basolateral membrane of human hepatocytes, can regulate the uptake of endogenous or exogenous substances, such as bile acids, thyroid hormones, repaglinide, and statins, by the liver. Therefore, the transport that it mediates may be key to rate-limiting the drug load of hepatobiliary elimination, thereby determining the trend of drug efficacy or toxicity[153,154]. Notably, in addition to possessing a certain liver-specific therapeutic activity, OATP1B1 participates in the cell damage and cholestasis induced by substrate drugs overloaded in hepatocytes, thereby mediating the occurrence of DILI[154]. Among them, bosentan, a targeted therapeutic drug for pulmonary arterial hypertension, is a substrate of OATP1B1. After OATP1B1 mediates the hepatic uptake of bosentan, it may induce the dysfunction of the canalicular bile salt export pump (BSEP). Finally, a large amount of bile acids accumulates in hepatocytes and serum bile salt concentration increases, that is, liver injury may occur concurrently. This phenomenon is the reason why liver blood function must be tested before patients are administered bosentan[155]. Therefore, further identifying OATP1B1 inhibitors with low biotoxicity to reduce the liver uptake of bosentan, thereby alleviating bosentan-mediated hepatotoxicity and OATP1B1-related DILI, is necessary. In rats treated with bosentan, the flavonoid BCA could markedly reduce bosentan exposure in the rat liver; reverse bosentan-induced cholestasis; and inhibit the content of serum total bile acid (TBA), a sensitive marker of liver injury, that is, by weakening the OATP1B1-regulated uptake of bosentan, thus showing remarkable anti-DILI effects[155]. In addition, a structure–activity relationship model constructed on the basis of common feature pharmacophores might achieve the structural optimization of flavonoid inhibitors and thus enhance the effectiveness of DILI mitigation and clarify whether the anti-OATP1B1 effect of flavonoids is determined by certain key pharmacophores. The calculation results of the pharmacophore model revealed that hydrogen bond acceptors and hydrogen bond donors were the main pharmacophores of OATP1B1 inhibitors. Among them, the hydrogen bond acceptors and hydrogen bond donors at the 4′-, 5-, and 7-positions were the joint driving forces that dominated the inhibitory function of OATP1B1. When these sites are occupied by other groups with larger steric hindrance, such as glucose, that is, when glycosylation is induced, it will damage the biological function of flavonoids against OATP1B1[156]. In summary, the research on the structure–activity relationships and activity prediction of various natural flavonoids, including BCA, show that deriving highly effective OATP1B1 inhibitors and applying them in the treatment of anti-DILI is of considerable potential.

6.4. Acetaminophen treatment-induced DILI

6.4.1. Gut–liver axis

Acetaminophen (APAP) is an over-the-counter medication with antipyretic and analgesic efficacy. However, its abuse can transform it into a foreseeable hepatotoxin. In the United States alone, APAP constitutes the primary cause of death in approximately 500 cases of DILI per year[157]. This situation is related to the conversion of high-dose APAP into the toxic compound N-acetyl-p-benzoquinone imine

(NAPQI) by the CYP450 family in hepatocytes. Notably, the endogenous GSH in the liver is often mobilized as a natural antidote for NAPQI. However, when this protective effect is overwhelmed by excessive NAPQI, NAPQI forms covalent bonds with proteins within hepatocytes, resulting in the synthesis of APAP protein adducts[158]. These end products ultimately lead to hepatocyte death by disrupting mitochondrial function. Currently, APAP-induced hepatotoxicity shows remarkable heterogeneity among different individuals, with female mice showing superior resistance to APAP-induced liver injury (AILI) than male mice; such superiority is determined by the differences in the abundances of their gut microbiota[159,160]. Therefore, Zeng et al. enhanced microbial isoflavone transformation in male mice through the male–female cohabitation of mice. They further discovered that co-housed male mice exhibited significantly down-regulated serum levels of ALT, AST, TNF-α, IL-6, monocyte chemoattractant protein-1, and monocyte chemoattractant protein-3 following excessive APAP treatment[38]. In consideration of the coprophagic behavior of mice and the mediating role of the gut–liver axis in the regulation of liver disease mechanisms, further fecal microbiota transplantation from control and co-housed individuals was conducted. The findings revealed that male mice receiving feces from co-housed male mice with females experienced significant negative regulatory effects on the aforementioned indicators. Microbial community analysis confirmed that *Rikenella microfusus* (*R. microfusus*) tended to have higher abundance in the co-housed male groups. Additionally, the further characterization of differential metabolites through HPLC analysis demonstrated that the cecal content and blood of male mice co-housed with female mice, as well as the feces and blood of men cohabiting with women, showed significantly increased BCA concentrations compared with those of controls. However, the microbial absorption of the isoflavone aglycone BCA from the diet depends on the activity of bacterial β-galactosidase (β-gal). The up-regulation of β-gal activity in the feces of male mice cohabiting with female mice and the induction of β-gal activity in the intestinal tract of recipient mice through the transplantation of cohoused fecal microbiota further revealed that the promoting effect of heterosexual cohabitation on the abundance of *R. microfusus* would achieve the dietary isoflavone BCA liberation and enhance its intestinal absorption through the action of β-gal. Among them, BCA treatment could markedly alleviate the APAP-induced death of hepatocytes; sterile inflammation; and the accumulation of the toxic products NAPQI and APAP protein adducts. In vitro, it inhibited the promoting effect of APAP on LDH release in primary mouse hepatocytes[38]. In summary, the anti-AILI effect of BCA is nearly identical to that of N-acetylcysteine, a known antidote for APAP and an antioxidant, further suggesting that the mechanism by which BCA alleviates APAP-induced hepatotoxicity may involve the reversal of oxidative stress. Specifically, BCA significantly inhibited the APAP-induced accumulation of MDA and ROS, as well as the depletion of GSH, and mitigated the reduction reactions of GPx4 and the cystine/glutamate antiporter xCT by APAP. Ultimately, the BCA-regulated inhibition of oxidative stress would further alleviate the excessive phosphorylation of apoptosis signal–regulated kinase 1, mitogen-activated protein kinase kinase 4, and c-Jun N-terminal kinase in the livers of APAP-treated mice. To elucidate the specific mechanism by which BCA targeted oxidative stress, the study found that

after crossing the hepatocyte membrane, BCA bound to pyruvate carboxylase (PC) and propionyl-CoA carboxylase subunit alpha (PCCA). The enrichment of PC and PCCA proteins in hepatocytes could supply fuel for the tricarboxylic acid (TCA) cycle, promoting the conversion of their enzymatic activity substrates, namely, pyruvate and propionyl-CoA, into oxaloacetate and methylmalonyl-CoA. The induced TCA cycle drove the production of glutamate, thereby providing additional available substrates for the *de novo* synthesis of GSH. Not only that, the low expression of PC and PCCA in the liver of patients with liver failure, along with the low content of BCA in feces, further illustrated the effectiveness of PC/PCCA and BCA in mediating the alleviation of acute liver injury (ALI)[38]. In summary, heterosexual cohabitation further promotes the intestinal absorption of BCA in an *R. microfusus*–dependent manner through inter-individual microbiome shifts, thereby targeting the metabolic regulation of the TCA cycle, ultimately improving AILI in male individuals.

6.4.2. Multi-target based polypharmacology prediction

Although single-target drugs possess high selectivity, they may not meet the therapeutic needs of DILI, which is induced via multiple targets, pathways, and levels. Therefore, the gradual transition from the old paradigm of one compound–one target to the development of multi-target drugs is necessary[161]. Among these, based on the characteristic of polypharmacology, one or more drugs can be applied to regulate multiple targets, this approach has become a new paradigm in drug discovery and design, aiding the exploration of multi-target drugs or effectively targeting the treatment of diseases mediated by multiple signaling cascades, and is expected to enhance the time efficiency of drug development[162,163]. Liu et al. constructed multi-target based polypharmacology prediction (mTPP), a computational model based on virtual screening and machine learning, and applied it in the discovery of multi-target drugs. By comparing the training models of four machine learning algorithms and integrating the data on the binding strength of a single component with multiple targets and the proliferation rate of compounds against APAP-induced injury human hepatocyte cell line L02, they found that the Gradient Boost Regression algorithm had better prediction reliability than the Multi-layer Perceptron, Support Vactor Regression, and Decision Tree Regressor algorithms[164]. Therefore, the mTPP model using Gradient Boost Regression algorithm with high precision could realize multi-target-based polypharmacology prediction. Liu et al. subsequently virtually screened the Traditional Chinese Medicine Chemistry Database for potential hepatoprotective compounds based on the mTPP model and predicted proliferation rates, ultimately identifying and predicting 20 traditional Chinese medicine components with potential activity against DILI. On the basis of oral bioavailability and drug similarity principles, chelerythrine and BCA were selected from 20 hepatoprotective components for in vitro activity validation in APAP-treated cell assays, reflecting the accuracy of the constructed mTPP model. The significant inhibition of the survival and proliferation rates of L02 cells incubated with APAP for 24 h were effectively reversed by the addition of the two candidate compounds, thereby corroborating that the model-predicted active compounds possessed anti-DILI effects[164]. In summary, given the reliability of the mTPP model in predicting multi-target drugs, its

application in pilot studies for screening multi-target drugs not only provides technical references for the development of polypharmacology but is suitable for discovering diverse hepatoprotective agents derived from traditional Chinese medicines. Such agents include the multi-target compound BCA, which has therapeutic potential against APAP-induced hepatotoxicity.

6.5. LPS/D-GalN treatment-induced hepatoxicity

Lipopolysaccharide (LPS) and D-galactosamine (D-GalN) induce large-area bleeding, necrosis, and neutrophil infiltration in liver tissue. ALI induced by their synergism is a classic model for simulating fulminant hepatitis or developing hepatoprotective agents[165–167]. The intraperitoneal injection of LPS and D-GalN up-regulated the content of hepatic MDA, the levels of the serum liver enzymes ALT and AST and proinflammatory factors IL-1β and TNF-α, and inflammatory infiltration might further drive liver necrosis and liver failure[165]. In a previously reported study, pretreatment with BCA 1 h before LPS/D-GalN challenge not only had a negative regulatory effect on the above indicators but could promote Nrf2 nuclear translocation and up-regulate the activities of HO-1 and the antioxidant enzymes SOD, CAT, and GPx in the cytoplasm[165]. In addition, the nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3 (NLRP3) inflammasome, a multi-protein complex in the cytoplasm, is assembled by the NLRP3 sensor, the apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) adapter protein, and the caspase-1 effector[168]. The NLRP3 inflammasome was overexpressed in the LPS/D-GalN-induced ALI model. However, BCA did not directly target the down-regulation of the activities of hepatic NLRP3, ASC, and caspase-1 induced by LPS/D-GalN. Instead, it interfered with the interaction among NLRP3, ASC, and caspase-1[165,169]. Not only that, thioredoxin-interacting protein (TXNIP) is an upstream effector molecule that activates the NLRP3 inflammasome under oxidative stress conditions. However, western blot analysis and immunoprecipitation results respectively showed that BCA could negatively regulate the LPS/D-GalN-induced TXNIP overexpression, and inhibit the activation of the NLRP3 inflammasome by interfering with the interaction between TXNIP and NLRP3[165].

6.6. CCl4 treatment-induced hepatoxicity

CCl_4, as a chemical inducer of hepatotoxicity, can trigger the up-regulation of serum AST, ALT, ALP, TC, TG, total bilirubin, urea, and creatinine content[170,171]. However, BCA pretreatment negatively regulated the above sensitivity indicators related to CCl_4-induced liver injury in a dose-dependent manner, particularly the TC level, indicating that BCA had antihypercholesterolemic activity. Meanwhile, CCl_4 treatment could down-regulate serum total protein (TP) and glucose concentration, whereas BCA could significantly restore serum TP level and improve CCl_4-induced hypoglycemia, demonstrating that it could act as a regulator of glucose homeostasis[172]. In addition, CYP2E1 participates in activating the metabolism of CCl_4 in liver microsomes. The subsequent hepatotoxic effects induced by CCl_4 can be triggered by its highly reactive metabolite, which has a certain affinity to O_2, and further triggers LPO after the reaction. This effect thereby further leads to the structural and functional impairment of cell membranes,

as well as triggers the imbalance of pro-oxidants and antioxidants in the body through the release of ROS[173]. However, the protective effect of BCA against CCl_4-induced hepatotoxicity could be attributed to its antioxidant activity, that is, its inhibition of the expression of CYP2E1 and reversal of the driving effect of CCl_4 on the down-regulation of the activities of antioxidant indicators, such as SOD, CAT, GPx, glutathione reductase (GR), and glutathione-*S*-transferase (GST)[172]. In addition, although CCl_4-induced trichloromethyl radical and oxidative stress are key mediators involved in early liver injury, the driving effect of activated Kupffer cells on inflammation often indirectly leads to secondary liver injury. After Kupffer cells are activated, they exert a pro-secretion effect on diverse inflammatory messenger molecules, illustrating that the proinflammatory cytokine TNF-α and prostaglandin synthase COX-2 are overexpressed in the hepatotoxic injury induced by CCl_4. However, BCA significantly inhibited inflammatory infiltration[172,174]. When CCl_4 induces liver injury and inflammatory responses, hepatocytes, Kupffer cells, and endothelial cells compensatorily produce a large amount of NO. Excessive NO may become a key mediator triggering oxidative damage in the liver, especially oxidative stress related to LPO, which corresponds to the overexpression of iNOS in the liver tissue of the CCl_4-induced ALI rat model. However, BCA significantly inhibited the CCl_4-driven overactivation of iNOS and the increase in NO production in vivo[172,175,176]. In addition, when hosts trigger immune defense mechanism to resist the invasion of foreign antigens, the overexpression of the leukocyte-common antigen (CD45) may induce misleading or fulminating immunity. CD45, a member of the membrane sialoglycoprotein family and a surface marker of immune cells, further participates in lymphocyte differentiation and activation and triggers excessive immunity. However, BCA intervention could reverse the up-regulation of CD45 expression in CCl_4 treatment–induced inflammatory liver disease[172]. In conclusion, these pharmacological benefits all indicate that the hepatoprotective mechanism of BCA is closely related to its bioactivities as an antioxidant, anti-inflammatory agent, and immunomodulator.

6.7. Heavy metal-induced hepatoxicity

6.7.1. Monotherapy/multidrug therapy against arsenic toxicity

Arsenic (As), a metalloid that is widely present in the natural environment and human activities, seriously threatens the health of approximately 200 million people worldwide, among which high-exposure populations from developing countries, such as India, Bangladesh, and Pakistan, are mainly affected[177–180]. Regional chronic As exposure can induce hepatomegaly, liver fibrosis, and liver tumors. Moreover, heavy metal–triggered oxidative stress will further up-regulate the risk of As exposure–related hepatotoxicity[181,182]. It is primarily manifested that during the metabolism of As in hepatocytes, various ROS, such as hydroxyl radicals, hydrogen peroxide, superoxide anions, and peroxyl radicals, are derived[183,184]. At this point, numerous antioxidant enzymes are mobilized to counteract the attack of free radicals, ultimately resulting in the depletion of antioxidant enzymes. Therefore, plant-derived antioxidant components are expected to replace metal chelators as candidate therapeutic agents against As toxicity[185]. Among them, BCA is a bioactive isoflavone compound. It has certain metal chelating properties and

antioxidant activities as a result of the orientation of its functional groups[37]. It could also inhibit LPO in the liver of As-exposed rats. This is attributed to the lipophilicity and membrane permeability of BCA, which can deposit in the lipid bilayer and thus reduce the damage caused by free radicals to the cell membrane structure[186]. Trivalent inorganic As is markedly more toxic than pentavalent inorganic As[187]. The toxicity-inducing mechanism of trivalent As may be related to its direct interaction with vicinal thiols and the biological ligands of sulfur-containing groups, participating in cellular oxidative stress and inducing the release of free radicals. In addition, As poisoning damages the integrity of the hepatic membrane structure; such damage manifests as the overexpression of the LPO indicator MDA. However, BCA showed strong antioxidant activity by relying on its pharmacological properties of free radical scavenging and membrane stability protection; it further up-regulated the activities of the antioxidant enzymes SOD and CAT and the content of plasma GSH[186]. Not only that, the heavy metal As has a strong affinity to sulfhydryl groups. Given that sulfhydryl groups are present in the chemical structures of the vast majority of lipid-metabolizing enzymes, BCA may target changes in lipid levels. Notably, BCA administration did not interfere with the content of phospholipids and free FAs in the liver. This effect might be attributed to the interaction between As and GSH but not to the interaction with lipid-metabolizing enzymes or inadequate supply of nicotinamide adenine dinucleotide phosphate[186]. Finally, Gyanendra Singh et al. also found that the bioaccumulation of As in the liver of *Swiss albino* mice with sodium meta-arsenite poisoning was significantly down-regulated under cotreatment with the flavonoids BCA, phloretin, and epigallocatechin-3-gallate. Among them, BCA could also up-regulate the levels of GSH, SOD, and GST, and inhibit the increase in the protein carbonyl content (PCC), LPO and its indicator MDA content induced by liver As exposure. The enhancement of this antioxidant defense capacity in hepatocytes might be related to the overexpression of Nrf2 signaling promoted by BCA, although the antioxidant molecule Nrf2 was measured only in kidney tissue[184]. In summary, considering the metal chelation and free radical scavenging properties of flavonoid antioxidants, such polyphenolic compounds may be developed as effective dietary products against liver As toxicity.

6.7.2. Multidrug therapy against chromium/arsenic toxicity

The heavy metal elements Cr and As are certified as Group 1 human carcinogens by the International Agency for Research on Cancer. Long-term exposure to Cr and As and their heavy metal compounds results in biotoxicity, with the liver, brain, and kidneys as the main target organs, thereby inducing malignant liver tumors, liver necrosis, diabetes, and other substantial organ damage or metabolic diseases[188–191]. Cr and As poisoning often attacks highly vascularized hepatic structures. For example, chronic Cr exposure can trigger LPO. By destroying the hepatic antioxidant system, it further leads to hepatotoxicity or liver necrosis[192]. The As toxicity accumulated in the liver induces metabolic transformation, thereby releasing ROS and triggering hepatocyte apoptosis[193]. Eventually, ROS with numerous unpaired electrons cause oxidative damage to biomacromolecules, such as DNA and proteins; the outcomes of such damage are cytotoxicity, genotoxicity, and hepatorenal toxicity and inflammatory injury[144,194]. In addition,

excessive ROS can activate the caspase cascade by targeting caspase-8 cleavage and its downstream effector caspase-3 and further promote nucleic acid degradation mediated by the activation of the apoptotic pathway[195]. However, Swapnil Tripathi et al. reported that the combined administration of the natural antioxidants phloretin (PHL), BCA, and coenzyme Q10 (CoQ10) inhibited the heavy metal load related to Cr and As in mouse liver tissue. It also reversed the increase in the levels of LPO, MDA, and the protein oxidation indicator PCC caused by the accumulation of Cr and As toxicity, and the decrease in the activities of CAT, GST, SOD, non-enzymatic antioxidant GSH, and total thiols (TT). In addition, the coadministration of PHL, BCA, and CoQ10 not only markedly alleviated the hepatotoxic injury concurrent with Cr and As co-stimulation, but also inhibited the DNA breakage driven by combined heavy metal poisoning, showing significantly up-regulated DNA yields and reduced DNA degradation rates. Not only that, the coadministration of PHL, BCA, and CoQ10 activated the SIRT1/Nrf2/HO-1/NAD(P)H:quinone oxidoreductase 1 (NQO1) signaling pathway. The activated Nrf2 further drives the synthesis of downstream phase II detoxifying enzymes and antioxidant enzymes, which will become the potential mechanism through which PHL, BCA, and CoQ10 manifest their antioxidant properties. Furthermore, their combined administration promoted the inactivation of the apoptotic effectors caspase-8 and caspase-3 by reversing the apoptosis triggered by oxidative stress. Therefore, the benefits of the coadministration of PHL, BCA, and CoQ10 in alleviating the hepatotoxicity caused by the co-exposure of Cr and As depends on their synergistic antioxidant and anti-apoptotic activities[144]. Notably, the protective effects of PHL, BCA, and CoQ10 against the oxidative damage caused by the accumulation of Cr and As in the mouse liver could be attributed to their ability to scavenge free radicals and stabilize membranes in a stoichiometric combination, as well as their excellent hydrogen-donating properties[144] (**Fig. 5**).

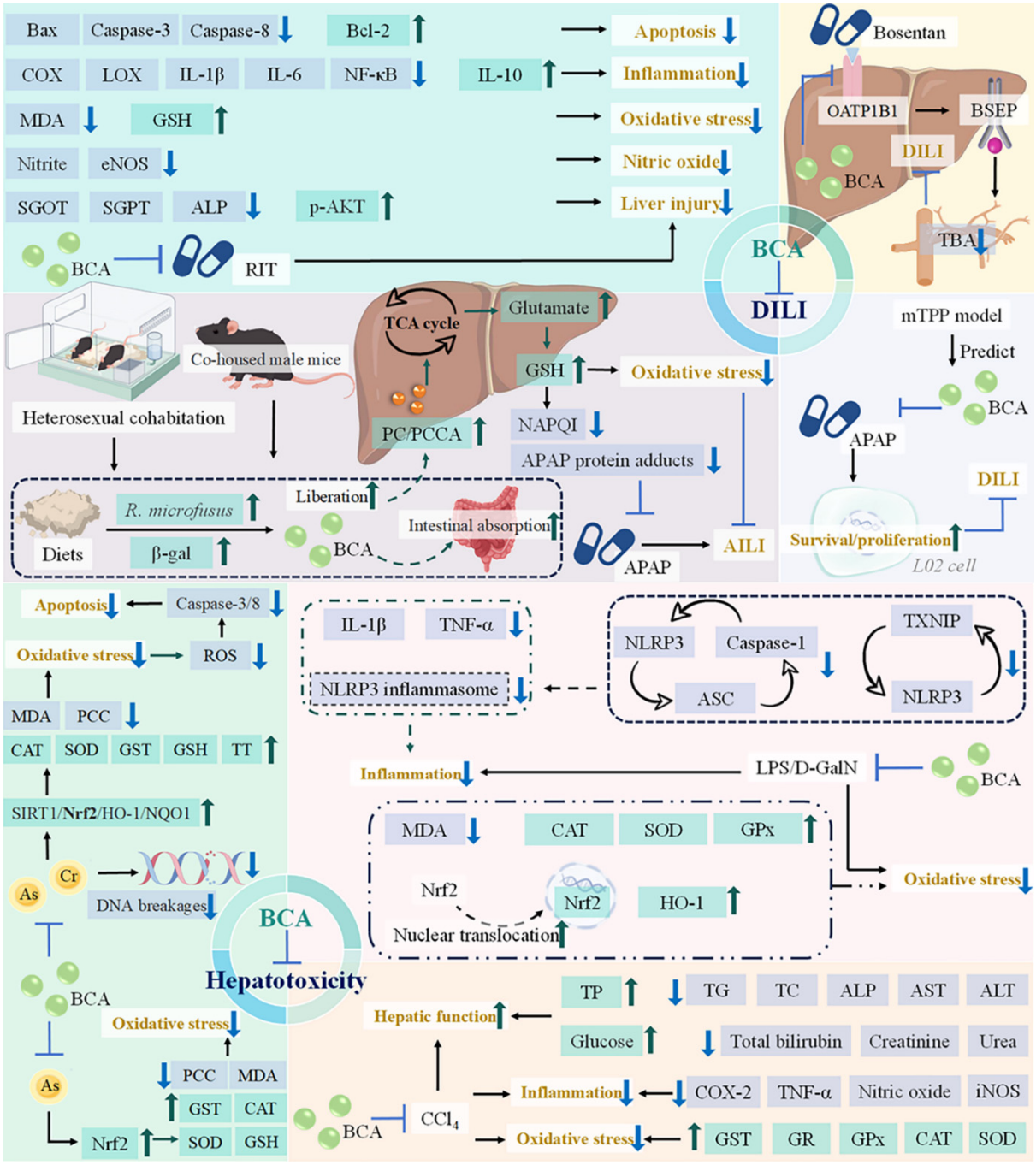

Fig. 5. Mechanisms of BCA in anti-DILI/hepatotoxicity. In combating DILI, BCA markedly alleviated RIT-induced liver injury by inhibiting apoptosis, inflammation, oxidative stress, and NO release. Secondly, heterosexual cohabitation could induce intestinal microbiome shifts in male individuals, wherein the up-regulation of *R. microfusus* abundance would further promote the dietary isoflavone BCA liberation through β-gal and enhance the intestinal absorption of BCA. Subsequently, BCA could supply fuel to the hepatic TCA cycle by binding to PC and PCCA to compensate for the massive consumption of the antioxidant GSH induced by APAP challenge and exert the detoxification effect of GSH on NAPQI and APAP protein adducts. Additionally, the hepatoprotective compound BCA, predicted by the mTPP model, could inhibit the APAP-induced impairment of the survival and proliferation rates of L02 cells. BCA could also alleviate the OATP1B1-mediated hepatic uptake of bosentan, thereby protecting BSEP function and down-regulating serum TBA content to counteract hepatic cholestasis. In terms of anti-hepatotoxicity, BCA could alleviate the interaction among NLRP3, ASC, and caspase-1, as well as the interaction between NLRP3 and TXNIP, thereby reducing the activation of the NLRP3 inflammasome, and further reversing LPS/D-GalN treatment–induced liver injury by driving the antioxidant effect mediated by the nuclear translocation of Nrf2. Furthermore, BCA could mitigate CCl_4-induced hepatotoxicity by down-regulating liver injury-related biomarkers and targeting oxidative stress and inflammation. Finally, BCA could also inhibit heavy metal poisoning–induced DNA breakage, and further reverse the inducing effects of accumulated heavy metal loads on oxidative stress in the liver by activating the SIRT1/Nrf2/HO-1/NQO1 signaling pathway or overexpressing the antioxidant molecule Nrf2. However, the elimination of ROS would also alleviate the apoptotic response related to caspase cascade activation.

7. The protective mechanisms of BCA against other liver lesions

Intracellular parasites, such as the opportunistic pathogen *Toxoplasma gondii*, can drive host macrophages to secrete proinflammatory cytokines and activate the MAPK signaling pathway, ultimately leading to portal vein dilatation and periportal lymphocyte infiltration in the livers of mice with chronic toxoplasmosis. However, BCA treatment remarkably alleviated necroinflammatory liver injury and portal inflammation and reversed the overexpression of hepatic TNF-α, IL-1β, and iNOS, thereby effectively reducing the parasitic cyst load in mice with experimental chronic toxoplasmosis[196]. Furthermore, Thuy-Lan-Thi Vo et al. identified five major soybean isoflavones, including BCA, in a 95% ethanolic extract of *C. cajan* roots (EECR95) through HPLC analysis. They discovered that EECR95 could significantly down-regulate the levels of liver function markers, such as cholesterol, TG, and GPT, in rat serum. Considering that EECR95 had no significant mutagenic effects on RAW 264.7, L-929, and HGF-1 cells at doses of up to 1,000 μg/mL, BCA could potentially be a safe, non-cytotoxic hepatoprotective component[197]. Lemon is one of the dominant fruit crops in the citrus plant (*Citrus aurantifolia*), with citrus peels also being the fruit by-product richest in flavonoid compounds[198]. HPLC analysis identified diverse flavonoid compounds, including BCA, in lemon peel extract. In Fructose–Streptozotocin rats, polyflavonoids extracted from lemon peel extract decreased serum liver enzyme activity, the hepatic LPO indicator thiobarbituric acid reactive substance, and the inflammatory markers TNF-α, NF-κB, IL-6, and alpha-fetoprotein. They also had inducing effects on serum albumin, a marker of liver synthetic function, and the contents of the hepatic antioxidants GSH and CAT. These effects may reflect the potential of BCA to resist diabetes-associated hepatic inflammation and oxidative liver damage[199]. Similarly, BCA could also alleviate diabetes-induced abnormal hepatic lipid profiles by inhibiting *de novo* lipogenesis and triggering FA oxidation. This process was not only associated with the up-regulation of hepatic antioxidant enzyme activity, but also involved BCA reversing the down-regulation of the expression of CPT and the overexpression of HMGCR, FAS, and ACC in diabetic rats induced by HFD and streptozotocin[200]. In the same diabetic rat model, researchers also found that BCA could alleviate the damage to the liver caused by glucotoxicity, and further restore hepatic glycogen content by regulating the activities of glycogen phosphorylase and glycogen synthase in liver[201]. In addition, Zhou et al. identified BCA as one of the important isoflavone components in chickpea flavonoids through ultra-performance liquid chromatography-tandem mass spectrometry analysis. They found that chickpea flavonoids could reverse the inhibitory effect of palmitic acid on the expression of insulin receptor substrate 1 and PI3K in IR-HepG2 cells, up-regulate the phosphorylation level of AKT, induce glucose consumption and glycogen synthesis, thereby activating the PI3K/AKT signaling pathway. Among these effects, the improvement of BCA on insulin signal disruption and IR in HepG2 cells might involve the metabolic regulation of aromatic amino acids, glutamine, linoleic acid, and palmitic acid[202]. **Table 1.**

Table 1. Therapeutic effects and underlying mechanisms of BCA in liver diseases.

Liver disease	Dosages	Models	In vivo/ in vitro	Effects and related mechanisms	Reference
HCC	25 mg/kg BCA and 7.5 mg/kg SB590885, i.p.; 75 μM BCA and 12 μM SB590885.	The HCC cell xenograft model in mice; HCC cell lines Bel-7402 and SK-Hep-1.	In vivo and in vitro	Suppressed the HCC cell proliferation by inhibiting the ERK MAPK and PI3K/AKT/mTOR signalling pathways, and triggered cell cycle arrest and apoptosis.	[57]
	15 μM BCA and 0.3 μM SOR.	HepG2, SNU-449 and HuH-7 cells.	In vitro	Induced synergistic cytotoxicity in HCC cells by interfering with the cell cycle, boosting the mitochondrial apoptosis, and impeding cell proliferation.	[70]
	20 μM of flavonoids.	Isoflavones were tested for their apoptosis induction activities in human hepatoma HuH-7 cells.	In vitro	Showed high apoptosis induction profiles in HuH-7 cells.	[73]
	20 μg of the combined isoflavones, subcutaneously; 20 μg/mL of each isoflavone alone or in combination.	Hep3B cells were injected subcutaneously into the backs of the mice; HepG2, Hep3B, HuH-7, PLC, and HA22T cells.	In vivo and in vitro	Suppressed the growth of human hepatoma cells, and induced apoptosis by activating caspase-3 and down-regulating Bcl-X_L and Bcl-2 expression.	[76]
	10 ppm, 20 ppm BCA, 10% miso or 2% NaCl, p.o.	DEN and fission neutron treatments-induced liver tumors in BCF_1 mice.	In vivo	Decreased multiplicities and sizes of liver tumors, inhibited DNA synthesis.	[78]
	100 nM of $1,25(OH)_2D_3$ and 50 μM of the phenolic substance.	HuH-7 cells were pre-treated with $1,25(OH)_2D_3$ and the phenolic substance and then subjected to both normoxic (21% O_2) and hypoxic conditions (1% O_2).	In vitro	Down-regulated the CYP24A1 expression to exert the anticancer potential of vitamin D.	[81]
MASLD	10/20/40 mg/kg, i.g.	High-fat and high-glucose diet-induced MASLD rat model.	In vivo	Improved the blood lipid metabolism and IR, and inhibited the release of hepatic inflammatory factors and intestinal endotoxins.	[88]
	HFD with 0.05% BCA, p.o.	HFD-induced obese mice.	In vivo	Regulated fatty acid oxidation, synthesis of phosphatidylcholine, and gluconeogenesis to improve hepatic steatosis and IR.	[90]
	1/40 μM.	HepG2 cells were incubated with estrogen or phytoestrogen.	In vitro	Exerted the cholesterol-lowering effect by activating hepatic LDLRs.	[94]

	30, 60, 120 mg/kg; 0.1 to 20 μM, 0.25 to 2.0 mM.	HFD-induced hyperlipidemia mouse model; RAW 264.7 macrophages, MCF-7 cells.	In vivo and in vitro	Inhibited hepatic lipid deposition by regulating HTGL and LPL activities, and improved hepatic antioxidant capacity.	[95]
	50 μM.	OA-induced steatosis in HepG2 cells.	In vitro	Demonstrated the effect of antihepatosteatosis by driving SIRT3/AMPK/ULK-1-mediated autophagy.	[100]
	6.25/10/12.5/25/50 $\mu mol \cdot L^{-1}$.	Murine AML12 cells, human HepG2 cells.	In vitro	Exhibited significant anti-inflammatory activity independent of transactivating FXR, and ameliorated lipid deposition.	[107]
Liver fibrosis	25/50 mg/kg, p.o.	TAA-induced liver cirrhosis in rats.	In vivo	Suppressed hepatocyte propagation and oxidative stress, and promoted free radical scavenging.	[26]
	50 mg/kg, i.p.	CCl_4-induced liver fibrosis in rats.	In vivo	Inhibited oxidative stress and the NF-κB-related inflammatory cascade, and the expression of profibrogenic factors.	[116]
	50 nmol/L.	PDGF-induced HSC-T6 proliferation.	In vitro	Inhibited the proliferative activity of HSC-T6 derived by PDGF, which might benefit in alleviating liver fibrosis.	[128]
	50 mg/kg, p.o.	*Schistosoma mansoni*-infected mice.	In vivo	Demonstrated the schistosomicidal, anti-inflammatory, antioxidant and anti-fibrotic effects, and inhibited CYP450 enzyme expression.	[132]
DILI/hepatotoxicity	20 mg/kg, i.p.; 20 μM.	APAP-induced ALI model in mice; primary mouse hepatocytes.	In vivo and in vitro	Promoted the intestinal absorption of BCA in an *R. microfusus*-dependent manner and provided fuel for the TCA cycle to enhance the resistance of male individuals to AILI.	[38]
	50 mg/kg, i.p.	Combined Cr and As intoxicated mice.	In vivo	Ameliorated heavy metal-induced hepatotoxicity, oxidative stress and apoptosis by activating the SIRT1/Nrf2/HO-1/NQO1 pathway.	[144]
	100 mg/kg, p.o.	RIT-induced hepatotoxicity in rats.	In vivo	Regulated the NF-κB/p-AKT signaling molecules and pro-apoptotic and anti-apoptotic factors, and inhibited oxidative stress.	[150]
	50 mg/kg, i.g.; 0 to 60 μM.	Bosentan-induced liver injury in rats; OATP1B1-HEK293 cells.	In vivo and in vitro	Demonstrated a notable in vitro and/or in vivo suppression of OATP1B1-mediated bosentan uptake, which could mitigate the cholestatic effects of bosentan in rats.	[156]
	12.5 μmol/L.	APAP-treated human L02 cells.	In vitro	Improved the survival and proliferation rates of APAP-induced injury cells.	[164]
	12.5/25/50 mg/kg, i.p.	LPS/D-GalN treatment-induced ALI in mice.	In vivo	Induced the Nrf2 pathway activation and inhibited NLRP3 inflammasome activity.	[165]
	5/25/50/100 mg/kg, i.p.	CCl_4-induced hepatotoxicity in rats.	In vivo	Suppressed inflammation and oxidative stress, and regulated immune responses.	[172]

	50 mg/kg, i.g.	$NaAsO_2$-induced As toxicity in the *Swiss albino* mice.	In vivo	Down-regulated the As bioaccumulation in liver, up-regulated the Nrf2 expression and antioxidant content.	[184]
	10/20/40 mg/kg, i.g.	As-induced hepatic injury and hepatotoxicity in rats.	In vivo	Alleviated hepatic oxidative injury, hematotoxicity and hepatotoxicity.	[186]
Other liver lesions	50 mg/kg, i.g.	A murine model with chronic toxoplasmosis.	In vivo	Down-regulated TNF-α, IL-1β, iNOS expression, alleviated necroinflammatory injury and parasitic cyst load in liver.	[196]
	0.2/1.0 g/kg of EECR95, p.o.; 10 to 1000 μg/mL of EECR95.	Administrated EECR95 to rats for 90 days; RAW 264.7, L-929, and HGF-1 cells.	In vivo and in vitro	Down-regulated the cholesterol, TG, and GPT contents in serum to exert hepatoprotective effects.	[197]
	400 mg/kg of lemon peel extract, p.o.	Fructose and streptozotocin treatments-induced T2DM in rats.	In vivo	Regulated the levels of biomarkers such as NF-κB, AFP, GSH, and TBARS to alleviate liver function impairment caused by diabetes.	[199]
	10 mg/kg, i.g.	HFD and streptozotocin treatments-induced diabetic rats.	In vivo	Improved abnormal hepatic lipid metabolism by regulating lipogenesis marker enzymes, and alleviated oxidative stress.	[200]
	10 mg/kg, i.g.	HFD and streptozotocin treatments-induced diabetic rats.	In vivo	Restored hepatic glycogen content by regulating the activities of carbohydrate-metabolizing enzymes, and alleviated the damage of glucotoxicity to the liver.	[201]
	10/20/40 μg/mL of chickpea flavonoids.	PA-induced insulin resistant HepG2 cells, and chickpea flavonoid incubation.	In vitro	Alleviated IR in HepG2 cells by activating PI3K/AKT signaling pathway, and inhibited oxidative stress and lipid metabolism disorders.	[202]

Abbreviations: $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D; AFP, alpha-fetoprotein; AILI, APAP-induced liver injury; AKT, protein kinase B; ALI, acute liver injury; AML12, alpha mouse liver 12; AMPK, adenosine monophosphate–activated protein kinase; APAP, acetaminophen; As, arsenic; BCA, biochanin A; CCl_4, carbon tetrachloride; Cr, chromium; CYP24A1, cytochrome P450 family 24 subfamily A member 1; CYP450, cytochrome P450; DEN, diethylnitrosamine; D-GalN, D-galactosamine; DILI, drug-induced liver injury; EECR95, 95% ethanolic extract of *C. cajan* roots; ERK, extracellular signal–regulated kinase; FXR, farnesoid X receptor; GPT, glutamic pyruvate transaminase; GSH, glutathione; HA22T, hepatoma cell line; HCC, hepatocellular carcinoma; Hep3B, hepatoma cell line; HepG2, hepatoma cell line; HFD, high-fat diet; HGF-1, human gingival fibroblasts; HO-1, heme oxygenase-1; HSC-T6, rat hepatic stellate cell line; HTGL, hepatic triglyceride lipase; HuH-7, hepatoma cell line; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; IR, insulin resistance; L02, human hepatocyte cell line; L-929, mouse fibroblasts; LDLR, low-density lipoprotein receptor; LPL, lipoprotein lipase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MASLD, metabolic dysfunction–associated steatotic liver disease; mTOR, mammalian target of rapamycin; $NaAsO_2$, sodium meta-arsenite; NaCl, sodium chloride; NF-κB, nuclear factor-kappa B; NLRP3, nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor 2; OA, oleate; OATP1B1, organic anion transporting polypeptide 1B1; OATP1B1-HEK293, human embryonic kidney 293 cells stably expressing OATP1B1; PA, palmitic acid; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol-3-kinase; PLC, hepatoma cell line; RIT, ritonavir; *R. microfusus*, *Rikenella microfusus*; SIRT3, sirtuin 3; SNU-449, hepatoma cell line; SOR, sorafenib; T2DM, type 2 diabetes mellitus; TAA, thioacetamide; TBARS, thiobarbituric acid reactive substance; TCA, tricarboxylic acid; TG, triglyceride; TNF-α, tumor necrosis factor-α; ULK-1, Unc-51-like kinase 1.

8. Foods or dietary supplements

Approximately 30%–70% of pathological populations and ≥10% of the general population have a history of taking herbal products[34]. Red clover is often prepared as an herbal preparation, and isoflavones derived from red clover have been widely sold on the market as neutraceutical extracts applied in commercial modes. Tablets (such as Promensil and Trinovin from Novogen), capsules, liquid preparations, and teas (such as Alvita, Botanic Choice, and TerraVita) are the main health product forms of such extracts available on the market[203]. Among them, each Promensil (Novogen, Inc., Samford, CT) tablet contains approximately 26 mg of BCA, and Promensil™ (Novogen, Sydney, Australia) is recommended to consume 1–2 tablets per day[31,204]. Therefore, an intake frequency of once a day is recommended if taking dietary isoflavone supplements chronically. Taking two tablets of 40 mg of isoflavone supplements daily results in blood drug concentrations similar to those in people with isoflavone-enriched diets[31].

At present, various plant-derived dietary supplements have been extensively developed and are favored by women consumers, especially postmenopausal women, to improve estrogen homeostasis. However, such nutritional supplements are not pure substances containing BCA alone but are instead mixtures of multiple functional components. Given their lack of awareness of drug–botanical interactions, women often inadvertently consume prescription drugs and plant-based dietary supplements simultaneously. Therefore, patients do not actively inform medical staff of this overlooked misunderstanding. This situation may ultimately be mainly attributed to the fact that plant-based dietary supplements, in contrast to newly developed prescription drugs, do not generally require the disclosure of potential pharmacokinetic information for approval, marketing, and application in clinical practice. Whether it involves multiple regulatory effects on drug transporters or the activities of drug-metabolizing enzyme remains unknown[32]. In addition, most developers have not provided intake dose ranges for reference. Therefore, blind intake only increases the isoflavone concentration in urine and cannot play a similar supplementing effect as a high-isoflavone diet in consumers' bodies[205]. This situation indicates that in the future, improving standardized operation of the BCA-based health product processing market should receive attention.

Notably, in addition to direct BCA ingestion, daily dietary supplementation based on chickpeas also indirectly increases the oral intake of BCA through edible plants. However, although chickpeas are widely accepted by the consumer market, their annual yield growth rate is only 1.3%, verifying the need for further improving their breeding and processing strategies[23]. Therefore, focusing on the development of transgenic chickpea cultivation technology to increase yield and combining it with mature food processing techniques will help improve the texture and taste of chickpeas and provide an important reference for alleviating yield pressure and broadening product differentiation. Examples of the above food processing techniques include grinding granular chickpeas into mash, chickpea flour, and other food raw materials, mixing them with nuts and grains, and then adding them to the production of cakes, biscuits, canned food, and bread or extracting high-purity chickpea protein concentrate and applying it in the production of plant-based protein drinks, protein powders, functional drinks, and meal replacement products.

9. The clinical translation of BCA

Currently, most of the studies on the hepatoprotective effects of BCA are based on rodent models, and tests on primates or human samples do not exist. However, compared with rodents, humans have a more efficient glucuronidation system, better ability to conjugate isoflavones, and lower proportion of unconjugated free aglycones in the blood circulation[40]. In addition, most in vivo animal and in vitro cell experiments used high intervention doses, which are dozens or hundreds of times higher than the average human isoflavone intake and are thus unscientific compared with the amount supplied by the human daily diet[40]. In summary, fundamental differences in the half-life, metabolic rate, and blood drug concentration of BCA exist between humans and experimental animals. Filling the gaps in clinical liver disease research is necessary, and such efforts cannot just be based on a mechanical repetition or dose extrapolation of animal data.

Searching http://clinicaltrials.gov/ for clinical trials related to BCA yielded only three results, and no clinical trials on liver disease are performing recruitment or have been completed. Currently, the clinical application of BCA is mostly limited to postmenopausal women and does not involve purified single phytochemicals. Instead, red clover extracts rich in isoflavones, such as BCA, are used for treatment. In a latest randomized controlled trial involving 75 postmenopausal women with dyslipidemia, 40 mg isoflavone red clover capsules were administered, with starch capsules of the same dose used as a placebo control. The results showed that the red clover intervention, when given twice a day for 3–6 months, provided considerable benefits in improving TC, TG, LDL cholesterol, and high-density lipoprotein cholesterol[206]. Additionally, a one-year double-blind intervention trial involved the daily administration of a novel red clover extract rich in 60 mg isoflavone aglycones and probiotics and supplementation with vitamin and mineral tablets. It was found that consuming the red clover extract twice a day for more than one year could effectively alleviate bone mineral density loss in postmenopausal women with osteopenia, improve estrogen deficiency–related osteoporosis, and induce a favorable estrogen metabolite profile, as well as equol production[207]. However, a double-blind randomized controlled trial involving 33 postmenopausal women with overactive bladder (OAB) reported negative results[208]. Although most clinical studies tend to confirm that local vaginal estrogen therapy improves OAB, the above trial found that taking red clover extracts twice daily for three months, which was equivalent to taking approximately 58.38 mg of isoflavones daily, had no significant effect on the urinary frequency symptoms or other voiding parameters of postmenopausal women with OAB[208,209]. This finding also indicates that BCA, as an estrogen analog, may have endocrine-disrupting properties[210]. However, the long half-life of BCA in human metabolism necessitates a sufficiently long study period to identify the toxic dose of BCA under real exposure. Furthermore, BCA has the biological nature of rapid demethylation into GEN. This characteristic casts doubt on whether the multi-target effect exerted after administration is derived from BCA itself or its metabolites. Not only that, given that BCA can induce the moderate and considerable inactivation of human CYP1A2 and CYP3A4/5 enzymes, respectively, it may pose potential risks of food–drug interactions as a

non-substrate of most CYP450 subunits that catalyze drug metabolism[7,28]. This situation is also a challenge that must be overcome for its clinical translation.

Finally, in contrast to those of most well-defined traditional hepatoprotective natural products, the efficacy of BCA has not yet been examined by expert panels. In the guidelines approved by the Russian Medical Scientific Society of Therapists and the Gastroenterology Scientific Society of Russia, silymarin was identified as a candidate therapeutic agent for ALD and MASLD. In addition, the expert panel approved 140 mg of the original silymarin three times a day as a safe hepatoprotective treatment dose[211]. However, although the level of clinical evidence for BCA is lower than that for silymarin, BCA has a higher safety factor than licorice (*Glycyrrhiza* spp.), an auxiliary therapeutic agent for primary liver disease. The US FDA has reported that licorice intake poses the risk of arrhythmia, hypertension, and hypokalemia; this risk is closely related to the mineralocorticoid effect of licorice and may even result in rhabdomyolysis[212,213]. Considering that the effect of licorice on blood pressure has a certain linear dose–response relationship, patients with hypertension taking BCA as a hepatoprotective therapeutic agent may effectively avoid the risk of severe persistent hypertension[213]. In addition, the synergistic effect of isoflavones and curcumin exerts significant antioxidant, anti-inflammatory, and anti-hepatic fibrosis activities[214]. However, similar to that of BCA, the oral bioavailability of curcumin is limited because of its biological characteristics of rapid metabolism, poor water solubility, and easy degradation within the gastrointestinal tract[215]. Notably, specific reports on how to use nanotechnology to improve the hepatic delivery of curcumin exist. For instance, curcumin delivery systems based on lipid-based carriers and polymeric nanoparticles have been developed, and curcumin derivatives have been prepared[216]. These approaches further help curcumin resist the metabolic pressure of rapid hydrolysis, thereby enhancing its targeting ability in the treatment of benign and cancerous liver lesions. Unfortunately, by contrast, most of the currently reported studies on improving the bioavailability of BCA have focused on enhancing its therapeutic efficacy against diseases, such as estrogen deficient-hypertension[30], pancreatitis[49], and melanoma[56]. They do not involve the development of delivery systems for optimizing its hepatoprotective activity. This situation indicates that certain resource investment is still required to achieve the transition of BCA from preclinical hepatoprotective research to clinical hepatoprotective application.

10. Conclusions and perspectives

In summary, BCA, as a natural hepatoprotective agent, possesses pharmacological activities, such as anti-cancer, anti-fibrosis, and anti-hepatotoxicity activities. First, BCA can synergistically enhance the anticancer efficacy of chemotherapeutic drugs. This ability is closely related to its inhibition of HCC cell proliferation and promotion of cell cycle arrest and apoptosis. Second, by regulating glycolipid metabolism homeostasis, activating autophagy, and FXR expression, BCA has become a botanical therapeutic agent for delaying the progression of MASLD. Third, BCA administration effectively alleviates the proliferation of fibrogenic effector cells in the liver by regulating profibrotic factors and activating anti-inflammatory and antioxidant signaling. Moreover, it can show bioactivity against *Schistosoma*-induced liver fibrosis.

Finally, BCA can be shuttled within the gut–liver axis, induce the TCA cycle, and serve as a detoxifying agent for heavy metal poisoning, drugs, or chemical toxin–induced liver injury by relying on its immunomodulatory activity and anti-apoptotic properties. Not only that, the multi-target therapeutic potential of BCA can be further predicted through virtual screening and machine learning. However, an overlap remains among targeted pathways across different liver diseases.

The integration of the literature revealed that most existing studies on the hepatoprotective effects of BCA tended to elucidate a single in vivo or in vitro mechanism, with combined research on both mechanisms being insufficient. Moreover, BCA is mostly present as an active component in mixed extracts or co-administered to intervene in liver diseases. This situation suggests that the introduction of gene-editing technology and integration of metabolomics or proteomics data will broaden the application prospects of BCA in the field of hepatology. At this stage, the bioavailability of BCA in the liver can be effectively improved by adding organic polymeric materials or designing novel targeted delivery systems. Secondly, natural product derivatives developed on the basis of technologies, such as metabolic engineering and synthetic biology, are conducive to offsetting the limitations of the bioavailability of their parent drugs, which will also promote the standardization of production and maximize the efficacy of natural product preparations containing BCA[217]. In addition, herbal medicines mediate the effects of interventions on the body's homeostasis through multiple complex regulatory mechanisms. This phenomenon potentially increases the challenges experienced by drug developers when quantifying their risk-to-benefit ratio before preparing new drugs[110]. Therefore, scientific data are lacking, and the medical market needs to strengthen regulatory measures for the development of BCA-rich dietary supplements to clarify whether drug–drug interactions tend to promote the optimization of bioavailability or weakening of hepatotoxicity. Furthermore, new strategies, such as drug–target–pathway networks, Intelligent Internet of Medical Things, and nanotechnology, may be beneficial to clarify the pathogenesis of diverse liver diseases. In the future, the exploration of the pharmacological mechanism and drug efficacy screening of BCA and its derivatives may draw on artificial intelligence technology. Not only that, further exploring the influence of variable factors, such as extraction technology, climate environment, geographical location, and planting conditions, on the content of active compounds and bioactivity of their main efficacy ingredients of natural products is necessary. This will improve the pharmacokinetic study of BCA and provide key pharmacological information for dosage scaling and human toxicity studies related to BCA[218]. Notably, most of the experimental studies on BCA in the field of hepatology focus only on MASLD, fibrosis, DILI/hepatotoxicity, cancer, and tumors, and works exploring the feasibility of BCA in alleviating other classical liver diseases, such as ALD, cholestatic liver injury, hepatic ischemia–reperfusion injury, and viral hepatitis, and in-depth research on relevant mechanisms do not exist. Therefore, the filling of relevant gaps in the literature will be of considerable practical importance in the future. Finally, because the current pharmacological research on BCA is mostly based on animal and cell line models with substantial differences from humans, and the potential mechanisms of the very low plasma concentration–effect

relationship shown by various natural active substances with clinical application value remain vague[219]. Therefore, further large-scale cohort studies combined with omics sequencing are needed to clarify the practical application value of BCA in human clinical practice and the pharmacodynamic fluctuations influenced by drug metabolism after in vivo binding with receptors. In short, the pharmacological information and various targeted pathways provided by this review may further demonstrate the effectiveness of BCA in the treatment of liver diseases and the necessity of optimizing the bioavailability of natural products.

Data Availability

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

Declaration of Competing Interest

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

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