Anticancer role of flubendazole: Effects and molecular mechanisms (Review)

XING XING[1*], ZONGNING ZHOU[2*], HONGWEI PENG[2,3] and SHAOPING CHENG[1]

[1]Department of Urology, The First Affiliated Hospital of Yangtze University, Jingzhou, Hubei 434000, P.R. China;
[2]Human Genetic Resources Preservation Center of Wuhan University, Wuhan, Hubei 430071, P.R. China;
[3]Hubei Key Laboratory of Urological Diseases, Zhongnan Hospital of Wuhan University, Wuhan, Hubei 430071, P.R. China

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Abstract. Flubendazole, an anthelmintic agent with a well-established safety profile, has emerged as a promising anticancer drug that has demonstrated efficacy against a spectrum of cancer types over the past decade. Its anticancer properties encompass a multifaceted mechanism of action, including the inhibition of cancer cell proliferation, disruption of microtubule dynamics, regulation of cell cycle, autophagy, apoptosis, suppression of cancer stem cell characteristics, promotion of ferroptosis and inhibition of angiogenesis. The present review aimed to provide a comprehensive overview of the molecular underpinnings of the anticancer activity of flubendazole, highlighting key molecules and regulatory pathways. Given the breadth of the potential of flubendazole, further research is imperative to identify additional cancer types sensitive to flubendazole, refine experimental methodologies for enhancing its reliability, uncover synergistic drug combinations, improve its bioavailability and explore innovative administration methods. The present review provided a foundation for future studies on the role of flubendazole in oncology and described its molecular mechanisms of action.

Correspondence to: Dr Hongwei Peng, Human Genetic Resources Preservation Center of Wuhan University, 169 Donghu Road, Wuhan, Hubei 430071, P.R. China
E-mail: iamhongwei@whu.edu.cn

Dr Shaoping Cheng, Department of Urology, The First Affiliated Hospital of Yangtze University, 55 Jianghan North Road, Shashi, Jingzhou, Hubei 434000, P.R. China
E-mail: csp810401@163.com

*Contributed equally

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Contents

1. Introduction to flubendazole

Flubendazole, also known as [5-(4-fluorobenzoyl)-1H-benzimidazole-2-yl]-carbamic acid methyl ester, is a benzimidazole carbamate anthelmintic drug which was first reported by Janssen Company (1) in the 1970s (Fig. 1). The fundamental mechanism of action of benzimidazole drugs against parasites is to bind tubulin in parasites, thereby disrupting the microtubule structure, blocking the normal function of the cytoskeleton, interfering with the normal movement and metabolism of the parasites, and ultimately leading to the death of the parasites (2-4).

Pharmacological safety and pharmacokinetics. As an antiparasitic drug, flubendazole has been used for the treatment of parasites in humans and animals for >40 years and its safety has been well established (1). Previous studies on pigs, hens, pheasants, dogs and rats have shown that flubendazole is a well-tolerated nonteratogenic drug (5,6). The low solubility of flubendazole in aqueous systems results in low absorption into the blood, which is partially responsible for the high safety profile of oral flubendazole for the treatment of intestinal parasitic infections in both animals and humans (1,7,8). Notably, a recent study reported that flubendazole affects the overall developmental processes and causes developmental neurotoxicity in zebrafish (9), which suggests that clinicians should be aware of the potential toxicity of flubendazole. The metabolites of flubendazole are predominantly reduced flubendazole, followed by hydrolyzed flubendazole, when it is administered intravenously or enterally (10,11). The pharmacokinetics of flubendazole have been previously summarized by Čáňová *et al* (1) and Chen *et al* (12).

Repurposing of flubendazole for anticancer effects. In 2010, Spagnuolo *et al* (13) first reported that flubendazole has anticancer

effects on leukemia and myeloma. Michaelis *et al* (14) reported the effects of flubendazole on the viability of a panel of cancer cell lines and showed that 117/321 (36.4%) of cancer cell lines had an IC_{90} value <1 μM, and 31/321 (9.7%) cell lines had an IC_{90} value between 1-5 μM. It was also reported that leukemia, multiple myeloma and neuroblastoma cells were the most sensitive to flubendazole. Currently, flubendazole has been reported to have anticancer effects on colon cancer, breast cancer, neuroblastoma, melanoma, glioma, esophageal cancer, lung cancer, prostate cancer and hepatocellular carcinoma (1,12,15). This drug repurposing approach can leverage the existing safety, dosage and pharmacokinetic data of drugs, shorten the time for new drug development, save substantial costs in the preclinical, phase I and phase II clinical stages, greatly reduce the cost of drug development, improve the efficiency of drug utilization and help to address the issue of drug resistance (16-18). The anticancer effects and mechanisms of flubendazole in various cancers have been summarized (Fig. 2; Table I).

2. Anticancer effects of flubendazole

Inhibition of microtubule structure and function. Microtubules, which are composed of α- and β-tubulin heterodimers, form slender, tube-like polymers that are crucial components of the cytoskeleton in all eukaryotic cells. They participate in processes such as cell division, intracellular transport, signal transduction and the maintenance of cell shape, polarity and integrity. During mitosis, the replicated chromosomes are separated into two identical sets before the cell divides into two daughter cells (19,20). The primary function of microtubules is to form a mitotic spindle that ensures the proper segregation and reassembly of chromosomes (19,20). The pivotal role of microtubules in mitosis makes them paramount targets for anticancer drugs (21). Flubendazole can specifically bind to tubulin and inhibit its polymerization, thereby exerting its anticancer effects (1,22).

In leukemia and myeloma, flubendazole induces cell death by binding to the polymerization site of tubulin, preventing the assembly of tubulin subunits and thus inhibiting the formation and stability of microtubules, which in turn affects cell cycle progression and induces mitotic catastrophe (13). Notably, flubendazole acts by binding to a site on tubulin that is similar to but distinct from the site targeted by vinblastine, which is why cells resistant to vinblastine due to overexpression of P-glycoprotein remain sensitive to flubendazole (13). Hou *et al* (23) reported that in breast cancer, the inhibition of tubulin polymerization by flubendazole leads to spindle abnormalities and the formation of monopolar spindles. These monopolar spindles fail to properly segregate chromosomes into two daughter cells, causing cell division failure and further inhibiting the proliferation of cancer cells. As microtubules are essential for spindle formation during the G2/M phase of the cell cycle, flubendazole inhibits the proliferation of cancer cells by suppressing tubulin polymerization and causing cell cycle arrest. By affecting tubulin polymerization, flubendazole may indirectly impact the self-renewal capacity of cancer stem cells, as the maintenance and function of these stem cells depend on normal cell cycle progression and cell division processes (19,23). The inhibition of tubulin polymerization may also affect epithelial-mesenchymal transition (EMT), a critical process through which cancer cells acquire invasive and metastatic capabilities. By disrupting the normal function of microtubules, flubendazole may inhibit the EMT process, thereby reducing the migration and invasion of cancer cells (22,23). Additionally, flubendazole enhances the cytotoxic effects of 5-fluorouracil (5-FU) and doxorubicin on breast cancer cells through cell cycle arrest caused by the inhibition of tubulin polymerization, which increases the sensitivity of cells to chemotherapeutic drugs (23). Furthermore, in colon cancer, melanoma and triple-negative breast cancer (TNBC), flubendazole can inhibit tubulin polymerization, induce mitotic catastrophe and cause cell cycle arrest (24-26).

Inducing cell cycle arrest. The cell cycle refers to the series of orderly processes that a cell undergoes from the end of one division to the end of the next. The cell cycle is primarily divided into two phases: Interphase and M phase, also known as mitosis. Interphase is further subdivided into the G1 phase, S phase and G2 phase, with the S phase being the period when DNA replication occurs. In the M phase, genetic material is accurately distributed to two daughter cells (27). During the G2 phase, the cell conducts a final check on DNA replication completed in the S phase, ensuring that no errors occur. At the G2/M transition, centrosomes, which are microtubule organizing centers present in animal cells, begin to replicate and migrate to opposite poles of the cell. Given that microtubules are key structures in the process of cell division and are responsible for forming the spindle apparatus and aiding in chromosome separation, factors affecting microtubule polymerization or function can lead to G2/M arrest (28).

In leukemia and myeloma cells, flubendazole induces G2/M cell cycle arrest and mitotic catastrophe by binding to tubulin and inhibiting microtubule polymerization (13). In breast cancer, flubendazole directly binds to the colchicine binding site of tubulin, preventing the polymerization of tubulin subunits, causing spindle abnormalities and leading to cell cycle arrest in the G2/M phase, thereby inhibiting the proliferation of cancer cells (23). In TNBC and HER2-positive breast cancer cells, flubendazole causes G2/M phase arrest of the cell cycle by binding to tubulin polymerization sites and affecting the levels of phosphorylated STAT3 (26,29). Flubendazole can suppress the proliferation of castration-resistant prostate cancer (CRPC) cells, induce expression of the p53 protein and increase the expression level of p21, also known as cyclin dependent kinase inhibitor 1A (CDKN1A), to inhibit the activity of cell cycle-related proteins such as Cyclin B1 and CDK1, thus preventing the cell from entering mitosis and causing the cell cycle to arrest at the G2/M phase (30). Additionally, flubendazole has been reported to induce G2/M phase cell cycle arrest in melanoma cells (25,31), intestinal cancer (32), glioma (33), glioblastoma multiforme (GBM) (34) and hepatocellular carcinoma (HCC) (35).

Inducing autophagy. Autophagy is a highly regulated intracellular process in which damaged proteins and organelles are degraded to maintain cellular homeostasis and provide energy resources. It serves a pivotal role in cellular stress, aging, the immune response and the progression of cancer (36). In cancer, autophagy serves a dual role. It serves as a survival mechanism for tumor cells, particularly in the context of

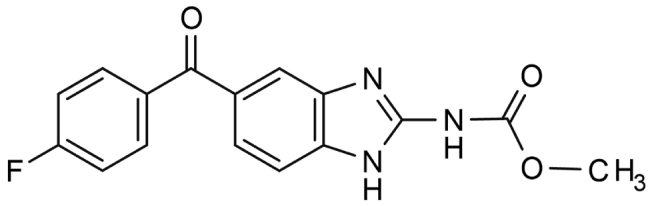

Figure 1. Structure of flubendazole.

nutrient scarcity by supporting uncontrolled cell proliferation. Conversely, if autophagy is hyperactivated or persists, it may lead to apoptosis (36,37). LC3 is essential for the formation and maturation of autophagosomes. There are two forms of LC3: LC3-I and LC3-II. LC3-II is generated through a process called lipidation, where LC3-I is conjugated to phosphatidylethanolamine to become LC3-II. This conversion is a key step in the elongation of the autophagosome membrane. Therefore, LC3-II is widely used in research as an indicator of autophagy activity (36,38).

Lin *et al* (39) and Xie *et al* (40) reported results on the efficacy of flubendazole in patients with colorectal cancer (CRC) and non-small cell lung carcinoma (NSCLC) in 2019 and 2021, respectively. *In vitro* experiments showed that flubendazole inhibited the expression of mTOR and P62, while upregulating the expression of Beclin-1 and LC3-I/II, thereby activating autophagy in both types of cancer. The activation of autophagy is associated with the induction of apoptosis in CRC cells, where activated autophagy can promote apoptosis. In *in vivo* tumor xenograft models, flubendazole reduced tumor volume and weight, and the experimental results showed that autophagy was activated (39-41). After treatment with flubendazole, the expression levels of the autophagy marker protein LC3-II in TNBC cells increased, the p62 protein was degraded and LC3 was converted from LC3-I to LC3-II (41). Furthermore, the formation of autophagosomes and autolysosomes induced by flubendazole was confirmed using the GFP/mRFP-LC3 dual fluorescence reporter system (41). Flubendazole impaired the permeability of the mitochondrial outer membrane in breast cancer cells, which was characterized by a decrease in the mitochondrial membrane potential, reduced ATP production and increased superoxide levels, leading to mitochondrial dysfunction. Flubendazole increased the expression of dynamin-related protein 1 (DRP1), leading to the aggregation of PTEN-induced putative kinase 1 (PINK1) and the mitochondrial translocation of Parkin, thereby promoting excessive mitophagy. Excessive mitophagy contributes to the mitochondrial damage and dysfunction induced by flubendazole, thus inhibiting the proliferation and migration of breast cancer cells (42).

Induction of apoptosis. Apoptosis is a form of programmed cell death that serves a crucial role in the development of an organism, the maintenance of tissue homeostasis and the elimination of damaged or aged cells. In the intrinsic pathway of apoptosis, pro-apoptotic proteins from the BCL-2 family form pores in the outer mitochondrial membrane, leading to increased mitochondrial membrane permeability and the subsequent release of cytochrome *c*. Cytochrome *c* then binds to the apoptotic protease activating factor-1, recruiting and activating procaspase-9. This activation subsequently triggers the activation of the effector caspases, caspase-3 and caspase-7, resulting in the process of apoptosis. In the extrinsic pathway, cell surface death receptors bind to their ligands, forming the death-inducing signaling complex, which activates caspase-8 and caspase-10, cleaves the BH3 interacting domain death agonist protein and promotes the release of cytochrome c, thereby intersecting with the intrinsic pathway (43,44).

In HER2-positive breast cancer cells treated with flubendazole, the expression levels of activated caspase-3, caspase-7 and caspase-8 increase, significantly increasing the proportion of apoptotic cells, particularly the number of late apoptotic cells (29). In CRC cells, flubendazole dose-dependently increases the activity of caspase-3, a key executor of apoptosis. The aforementioned study also showed that flubendazole promotes apoptosis by activating autophagy. The apoptotic effects observed *in vitro* were confirmed by *in vivo* experiments. In a xenograft model, flubendazole significantly reduced tumor volume and weight and an increase in the number of apoptotic cells was detected using TUNEL staining (39). In NSCLC, flubendazole reduces the expression of BCL-2 by activating the JNK pathway. Decreased expression levels of BCL-2 leads to the release of Beclin-1 from the BCL-2-Beclin 1 complex, thereby triggering autophagy, indicating that flubendazole can induce apoptosis in NSCLC cells by activating autophagy (40). In TNBC cells, an increase in the proportion of apoptotic TNBC cells induced by flubendazole was confirmed using the TUNEL assay and flow cytometry. An increase in the cleavage of caspase-3, upregulation of Bax and downregulation of BCL-2, as detected by immunoblotting analysis, suggested that flubendazole induced apoptosis (26,41).

Inhibition of cancer stem cell (CSC) properties. CSCs are a unique subset of cells that serve a crucial role in cancer initiation, progression, metastasis, therapeutic resistance and recurrence (45,46). These cells possess characteristics akin to those of stem cells, such as self-renewal and multilineage differentiation abilities, distinctive cell division patterns, metabolic phenotypes and robust resistance to conventional anticancer therapies (45,46). Studies on the inhibitory effects of flubendazole on the stemness of CSCs have primarily been conducted in the context of breast cancer (23,26,29). Breast CSCs (BCSCs) are a subpopulation of breast cancer cells characterized by high tumorigenicity and self-renewal. Key BCSC biomarkers include CD44, CD24, CD49f and aldehyde dehydrogenase (ALDH) (46,47). CD44 is a transmembrane glycoprotein which is pivotal for cell survival and pluripotency maintenance in BCSCs. CD24 is a sialomucin with low or absent expression levels in BCSCs and is implicated in cell adhesion and metastasis. CD49f, a laminin-binding glycoprotein, is crucial for cell-extracellular matrix interactions. ALDH is an enzyme associated with CSC self-renewal, differentiation and resistance to therapy, which serves an important role in cancer metabolism and progression.

In 2015, Hou *et al* (23) reported that flubendazole suppressed the expression of genes associated with self-renewal in breast cancer, including c-Myc, Sox2, Oct-4 and Nanog, and reduced the proportion of the $CD44^{high}/CD24^{low}$ subpopulation, suggesting that flubendazole decreased the self-renewal capacity of BCSCs. The ability to form mammospheres is

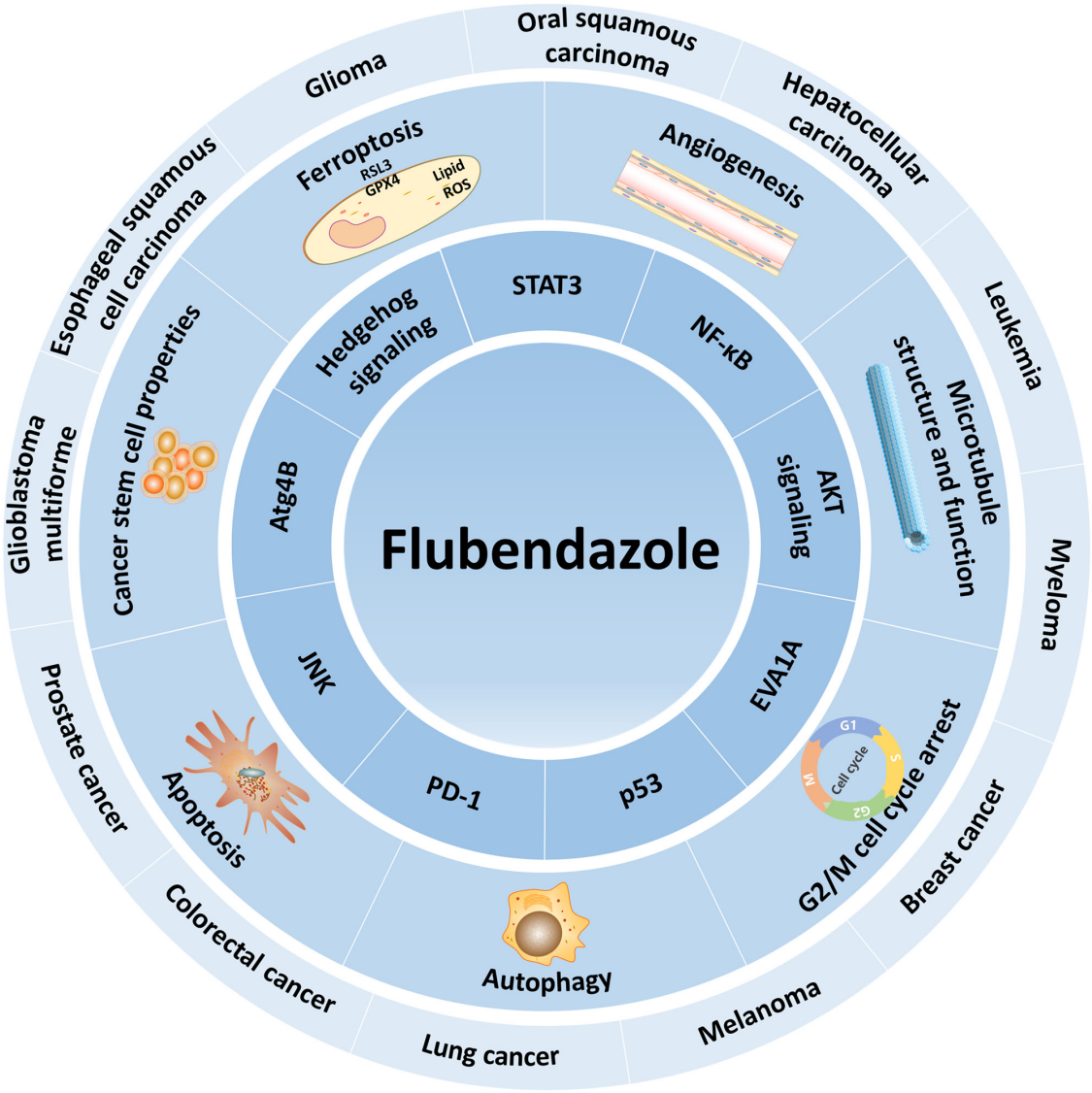

Figure 2. Molecular mechanisms and anticancer targets of flubendazole in different types of cancers. Atg4B, autophagy-related protein 4B; PD-1, programmed cell death protein-1; EVA1A, Eva-1 homolog A; ROS, reactive oxygen species; GPX4, glutathione peroxidase 4; RSL3, RAS-selective lethal 3; CSC, cancer stem cell.

an indicator of the self-renewal capacity of BCSCs. After treatment with flubendazole, breast cancer cells exhibited reduced ALDH1 activity, a decrease in the proportion of $CD44^{high}/CD24^{low}$ phenotype cells and a decrease in mammosphere formation capacity, indicating the suppression of stem cell properties in BCSCs (29). Another study focused on TNBC also reported that flubendazole could significantly affect BCSC-like properties, including reducing the proportions of $CD24^{low}/CD44^{high}$ and $CD24^{high}/CD49f^{high}$ subpopulations, decreasing ALDH1 activity and reducing the capacity for mammosphere formation (26).

Ferroptosis induction. Ferroptosis, an iron-dependent form of nonapoptotic cell death, is characterized by uncontrolled lipid peroxidation within the cell, ultimately leading to the rupture of the cell membrane. Ferroptosis involves multiple key molecules, including glutathione peroxidase 4 (GPX4), glutathione (GSH) and solute carrier family 7 member 11 (SLC7A11). SLC7A11 encodes a cystine/glutamate transporter responsible for transporting cysteine into the cell. Cysteine is a key precursor for the synthesis of GSH, which is one of the primary antioxidants in the cell. GPX4 is an essential antioxidant enzyme that utilizes GSH as a cofactor to reduce lipid hydroperoxides, protecting the cell membrane from oxidative damage. When the function of GPX4 is compromised or the expression levels of GSH decrease, phospholipids in the cell membrane become susceptible to lipid peroxidation catalyzed by iron ions, leading to destruction of the cell membrane and cell death (48-50).

In CRPC, flubendazole can induce p53 protein expression. p53 binds to the promoter region of SLC7A11, inhibiting its transcription, which results in decreased expression of the SLC7A11 protein. The reduced expression level of the SLC7A11 protein results in a decreased ability of the cell to take up cysteine, which in turn leads to a decrease in GSH levels. A decrease in GSH levels leads to a reduction in the activity of GPX4. A decrease in GPX4 activity results in the accumulation of lipid hydroperoxides, which can react with

Table I. Summary of anticancer targets and mechanism molecules of flubendazole in different cancers.

Cancer type	Experimental model	Concentration or dose	Synergetic drug	Effects/targets	Key molecules	(Refs.)
Leukemia and myeloma	OCI-AML2 and OPM2 cells; xenograft model	0.5, 1 or 2 μM; 20 or 50 mg/kg (i.p.)	Vinblastine	Microtubule structure and function, G2 cell cycle arrest and mitotic catastrophe	Tubulin polymerization	(13)
Breast cancer	MDA-MB-231, BT-549, SK-BR-3 and MCF-7 cells; xenograft model	0.125, 0.25 or 0.5 μM; 25 mg/kg (i.p.)	Fluorouracil and doxorubicin	$CD44^{high}/CD24^{low}$ subpopulation, self-renewal related genes, EMT and G2/M cell cycle arrest	Tubulin polymerization	(23)
Breast cancer	MDA-MB-231, Hs578T, BT-549 and 4T1 cells; xenograft model	0.1, 0.25 or 0.5 μM; 10 mg/kg (i.p.)	N/A	Apoptosis, G2/M cell cycle arrest, cancer stem cell-like properties and angiogenesis	STAT3	(26)
Breast cancer	BT474, SKBR3, JIMT-1 and MDA-MB-453 cells; xenograft model	0.1, 0.25 or 0.5 μM; 20 mg/kg (i.p.)	N/A	G2/M cell cycle arrest, apoptosis, HER2/HER3 heterodimerization, cancer stem cell-like properties and trastuzumab-resistance	HER2/AKT signaling	(29)
Breast cancer	DA-MB-231 and MDA-MB-468 cells; xenograft models	0.25, 0.5, 1 or 2 μM; 10, 20, or 40 mg/kg (i.p.)	N/A	Autophagy and apoptosis	EVA1A	(41)
Breast cancer	MDA-MB-231 and MCF-7 cells	0.25, 0.5 or 1 μM	N/A	Mitochondrial outer membrane permeability and mitochondrial function	EVA1A	(42)
Breast cancer	MCF-7 and MDA-MB-231 cells; xenograft model	1.5, 0.5, 1 or 2 μM; 15 mg/kg	Paclitaxel	Aberrant mitosis and apoptosis	HIF1α/PI3K/AKT signaling	(66)
Breast cancer	MDA-MB-231 cells	750 nM	N/A	Autophagy and reactive oxygen species production	Atg4B	(76)
Melanoma	A-375, BOWES and RPMI-7951 cells	1 μM	N/A	G2/M cell cycle arrest, microtubular damage, mitotic catastrophe and apoptosis	Tubulin polymerization and p53	(25)
Melanoma	A-375, BOWES and RPMI-7951 cells; 3 patients with melanoma	1 μM	N/A	G2/M cell cycle arrest, mitotic catastrophe, apoptosis and autophagy	JNK and Noxa	(31)
Melanoma	MDA-MB-435 cells; xenograft models	1 μM; 200 mg/kg (i.p.) or 20 mg/kg (i.t.)	N/A	Angiogenesis, PD-1 and myeloid-derived suppressor cells	STAT3	(51)
Melanoma	B16F10 and Jurkat cells; xenograft models	10 μM; 200 mg/kg (i.p.)	N/A	PD-1 expression, genes in cancer-associated pathways and immunological signature gene sets	PD-1	(67)
Colon cancer	SW480 and SW620 cells	1 or 2 μM	N/A	Microtubule organization, tubulin content, mitotic catastrophe and senescence	Tubulin polymerization	(24)
Colon cancer	HCT116, RKO and SW480 cells; 12 patients with colorectal cancer	0.3, 0.6 or 1.2 μM; 10 or 30 mg/kg (i.p.)	5-fluorouracil	Autophagy, apoptosis and nuclear translocation of STAT3	STAT3	(39)

Table I. Continued.

Cancer type	Experimental model	Concentration or dose	Synergetic drug	Effects/targets	Key molecules	(Refs.)
Colon cancer	SW480 and SW620 cells	1 μM	N/A	Cell adhesion and migration	NF-κB	(60)
Intestinal cancer	SW480, SW620, HCT8 and Caco-2 cells	0.2, 0.25, 0.3, 0.4, 0.5 or 1 μM	Paclitaxel	G2/M cell cycle arrest	N/A	(32)
Prostate cancer	PC-3, DU145 and RWPE-1 cells; xenograft model	0.1, 0.5 or 1 μM; 10 mg/kg (i.p.)	5-fluorouracil	G2/M cell cycle arrest and ferroptosis	p53	(30)
Oral squamous carcinoma	PE/CA-PJ15, DOK, H376 and GF cells	0.1 or 0.25 μM	N/A	Proliferation, migration and cadherin switching	N/A	(22)
Glioma	SF-268 and T-98G cells; xenograft model	0.25 or 0.5 μM; 25 mg/kg (i.p.)	N/A	G2/M cell cycle arrest and apoptosis	p53	(33)
Glioblastoma multiforme	U87-MG and U251-MG cells; xenograft model	0.125, 0.25 or 0.5 μM; 12.5, 25 or 50 mg/kg (i.p.)	N/A	DNA synthesis, G2/M cell cycle arrest, pyroptosis and apoptosis	p53 and NF-κB	(34)
Hepatocellular carcinoma	SNU449, PLC/PRF/5, Hep3B, HepG2, Huh7, MHCC-97H, MHCC-LM3, HCC-LY10 and HEK-293T cells; xenograft model	0.25, 0.5 or 1 μM; 40 mg/kg (i.p.)	Lenvatinib	Apoptosis and G2/M cell cycle arrest	Hedgehog	(35)
Lung cancer	H460, A549, PC-9 BEAS-2b and human umbilical vein endothelial cells; xenograft model	0.5, 1 or 2 μM; 10 or 20 mg/kg (i.p.)	N/A	Apoptosis, nuclear translocation of STAT3 and autophagy	STAT3	(40)
Esophageal squamous cell carcinoma	EC9706 and TE1 cells	1, 2 or 4 μM	Doxorubicin	Apoptosis and cytotoxicity of doxorubicin	NF-κB	(61)

NA, not applicable; i.p., intraperitoneal; i.t., intratumoral; EVA1A, eva-1 homolog A; HIF1α, hypoxia-inducible factor 1α; PD-1, programmed cell death protein-1; HER3, human epidermal growth factor receptor 3.

polyunsaturated fatty acids in the cell membrane, initiating lipid peroxidation. The accumulation of lipid peroxidation ultimately leads to damage to the cell membrane and cell death, resulting in ferroptosis (30).

Inhibition of angiogenesis. Treatment of TNBC cells with flubendazole inhibits the activation of STAT3, which consequently leads to a reduction in cancer angiogenesis. This is associated with a decrease in microvessel density and the expression level of VEGF. In a xenograft model of TNBC, flubendazole suppressed tumor growth, angiogenesis and metastasis to the lungs and liver, which coincided with reduced levels of MMP-2 and MMP-9 in circulating blood (26). In primary tumors formed by subcutaneous inoculation of melanoma MDA-MB-435 cells in xenograft models, flubendazole almost completely inhibited the expression of the cancer endothelial cell marker CD31, indicating that flubendazole is a potent inhibitor of cancer angiogenesis. STAT3 serves a role in regulating angiogenesis and flubendazole achieves its antiangiogenic effects through the inhibition of STAT3 (51).

3. Molecular mechanisms of the anticancer activity of flubendazole

STAT3. STAT3 is a latent cytoplasmic transcription factor that serves a pivotal role in various cellular processes, including cell proliferation, differentiation, inflammation, apoptosis, angiogenesis and immune responses (52). Under normal physiological conditions, STAT3 activation is tightly regulated. However, STAT3 is constitutively activated in many types of cancers, contributing to cancer growth and metastasis by regulating the expression of various target genes involved in cell survival, oncogenesis, cancer progression and stemness (53,54). The multifaceted role of STAT3 in cancer includes acting both as an oncogene and a cancer suppressor factor, depending on the specific cellular microenvironment and cancer type (55,56).

In breast cancer cells, flubendazole significantly inhibits the activation of STAT3, promoting TNBC cells to exhibit a marked increase in apoptosis, reducing the stem-like characteristics of BCSCs, decreasing the expression of VEGF and suppressing the process of EMT. In *in vivo* experiments, flubendazole significantly reduced the number of lung and liver metastatic foci by inhibiting STAT3 activation and decreasing the levels of MMP-2 and MMP-9 in circulating blood (26). In primary tumors formed by subcutaneous inoculation of melanoma MDA-MB-435 cells in xenograft models, flubendazole reduced the phosphorylation of STAT3, particularly at the Tyr705 site, exerting antiangiogenic effects by inhibiting STAT3 (51). In *in vivo* experiments, flubendazole completely inhibited programmed cell death protein-1 (PD-1) expression in cancer tissue by inhibiting STAT3 without affecting PD-L1 levels (51). Myeloid-derived suppressor cells (MDSCs) are a group of myeloid cells in the cancer microenvironment that inhibit immune responses and promote cancer progression through various mechanisms, facilitating immune evasion and metastasis. Flubendazole reduced the number of MDSCs in melanoma tissue, indicating that flubendazole may weaken the immunosuppressive effect of cancer by reducing the number of MDSCs, thereby contributing to the anticancer immune response (51). A previous study showed that flubendazole also affects the function of MDSCs by inhibiting the activity of STAT3 (51). In human CRC, flubendazole can block the activation and nuclear translocation of STAT3 induced by IL-6, leading to the inhibition of the transcription of STAT3 target genes, such as MCL1 apoptosis regulator, BCL-2 family member, VEGF and baculoviral IAP repeat containing 5. These genes are closely related to the antiapoptotic characteristics of cancer cells, angiogenesis and metastasis (39). In NSCLC, flubendazole can block the phosphorylation of STAT3 in a dose- and time-dependent manner, thereby regulating the transcription of STAT3 target genes and exerting anticancer effects through apoptosis and autophagy. As observed in CRC, flubendazole treatment of NSCLC can also inhibit the phosphorylation and nuclear localization of STAT3 induced by IL-6, thus inhibiting the activation of STAT3 and reducing the expression levels of VEGF and MCL-1 proteins related to STAT3, thereby affecting cancer angiogenesis and cell survival (40).

NF-κB. NF-κB is a pivotal transcription factor that is ubiquitously present in various types of cells and comprises five family members (p65, RelB, c-Rel, NF-κB1 and NF-κB2) (57). NF-κB serves a crucial role in controlling cell proliferation, inflammation, cellular stress responses, immune responses and apoptosis (57,58). NF-κB typically binds to the inhibitory protein IκB in the cytoplasm. Upon cellular reception of external signals, such as inflammatory factors, stress or injury, the IKK complex is activated and phosphorylates IκB, leading to its degradation and the release of NF-κB into the nucleus, where it activates the expression of target genes (57,58). NF-κB is a complex regulatory factor that may serve different roles at various stages of cancer development. It directly participates in the development of cancer by promoting the proliferation of cancer cells, inhibiting apoptosis, promoting angiogenesis and stimulating invasion and metastasis (57-59). In particular, it enhances the migratory and invasive capabilities of cancer cells by inducing EMT, thereby facilitating distant metastasis (57-59).

In CRC, fenbendazole not only affects cell adhesion and migration but also inhibits the phosphorylation of NF-κB p65, a key transcription factor associated with inflammation, survival, proliferation and metastasis. By employing RNA silencing technology to knock down NF-κB p65 in SW620 cells, the inhibitory effect on multiple cancer metastasis markers, such as intercellular adhesion molecule 1, epithelial cell adhesion molecule, integrin α5, β1 and α-tubulin, was enhanced. Fenbendazole suppresses the metastasis of CRC cells by inhibiting the activation of NF-κB p65, thereby reducing the expression levels of proteins related to cancer metastasis (60). In esophageal squamous cell carcinoma (ESCC), fenbendazole can inhibit the activation of IKK and decrease the phosphorylation of NF-κB p65. After 24 h of treatment with fenbendazole, it can induce the cleavage of poly ADP-ribose polymerase (PARP) and reduce the expression of the antiapoptotic protein BCL-2 while upregulating the expression of the proapoptotic protein Bim, ultimately leading to the apoptosis of ESCC cells. When used in combination with the chemotherapeutic drug doxorubicin, fenbendazole may enhance the toxicity of doxorubicin in ESCC cells by inhibiting the NF-κB

signaling pathway, demonstrating a synergistic effect (61). NF-κB is hyperactivated in GBM, and fenbendazole can trigger pyroptosis in GBM cells through the NF-κB/NLR family pyrin domain containing 3 (NLRP3)/gasdermin D (GSDMD) pathway (34). Pyroptosis is a form of programmed cell death closely related to inflammatory processes and is characterized by cell swelling, cell membrane rupture and the release of proinflammatory cytokines, such as IL-1β and IL-18. NF-κB mainly promotes the production of proinflammatory factors, such as pro-IL-1β, pro-IL-18, NLRP3 and caspase-1, which serve key roles in pyroptosis. In particular, NF-κB can promote the activation of the NLRP3 inflammasome, which in turn promotes the release of IL-1β and IL-18 mediated by caspase-1 and the cleavage of GSDMD (34). Therefore, fenbendazole can not only trigger pyroptosis in glioblastoma cells, but also induce mitochondria-dependent apoptosis (34).

AKT signaling. The PI3K/AKT/mTOR signaling pathway serves a crucial role in promoting cell survival, growth and cell cycle progression, with extensive cross-regulation with other cellular signaling networks. PI3K catalyzes the conversion of phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate (PIP3). The generation of PIP3 is a prerequisite for activation of the AKT protein. AKT is present in the cytoplasmic matrix in an inactive form. Upon activation of PI3K and production of PIP3, AKT is recruited to the plasma membrane and activated through phosphorylation by pyruvate dehydrogenase kinase 1 and mTOR complex 2. Activated AKT can migrate to the cytoplasm and nucleus, phosphorylating multiple substrate proteins that regulate key cellular functions such as proliferation, survival and metabolism. mTOR is a crucial regulator of cell growth and proliferation and primarily controls protein synthesis, cytoskeletal organization, cell growth and cell metabolism (62-64).

Flubendazole can significantly reduce the phosphorylation levels of HER2 and human epidermal growth factor receptor 3 (HER3) in HER2-positive breast cancer cells, thereby decreasing the levels of phosphorylated AKT, indicating that flubendazole may inhibit the PI3K/AKT signaling pathway by suppressing the formation of HER2/HER3 heterodimers and the activation of AKT. p95HER2 is a C-terminal truncated form of the HER2 receptor with tyrosine kinase activity that is associated with resistance to trastuzumab, also known as Herceptin. Flubendazole can significantly reduce the expression level of p95HER2, contributing to the inhibition of the AKT signaling pathway (29). Hypoxia-inducible factor 1α (HIF1α) is a key transcription factor in the cellular response to hypoxic environments that activates multiple genes associated with angiogenesis, glycolysis and cell survival. In cancer, the abnormal expression of HIF1α is closely related to the aggressiveness, metastasis and therapeutic resistance of cancers (65). RNA sequencing (RNA-seq) showed changes in a number of pathways controlling cellular metabolic processes in MDA-MB-231 breast cancer cells treated with flubendazole combined with paclitaxel, particularly a significant reduction in the expression levels of HIF1α, phosphorylated (p)-PI3K, p-AKT and p-mTOR. This suggests that flubendazole may produce a synergistic anticancer effect by reducing the expression level of HIF1α and inhibiting the PI3K/AKT signaling pathway. Furthermore, in paclitaxel-resistant breast cancer cells (MCF-7/PTX cells), treatment with flubendazole inhibited the PI3K/AKT pathway and reduced the level expression of HIF1α, demonstrating the potential to reverse paclitaxel resistance in breast cancer treatment (66). In addition, the expression of a number of genes related to immune responses, cellular metabolism and cell signaling was altered in human Jurkat cells treated with flubendazole. In particular, flubendazole upregulated genes associated with the PI3K/AKT signaling pathway, which is closely related to the effector function of T cells (67).

Eva-1 homolog A (EVA1A). EVA1A is a transmembrane protein associated with lysosomes and the endoplasmic reticulum that serves a crucial role in regulating autophagy and apoptosis. In cancer cells, EVA1A can interact with autophagy-related proteins, such as autophagy related 16 like 1, to promote the formation of autophagosomes, thereby inhibiting the proliferation of cancer cells. Furthermore, EVA1A can also induce cancer cell apoptosis by affecting the activation of caspase cascades (68).

Zhen *et al* (41) reported that flubendazole induced apoptosis and autophagy in TNBC cells. RNA-seq analysis showed that the expression of EVA1A was upregulated in TNBC cells after treatment with flubendazole. Silencing EVA1A with small interfering RNA significantly restored the reduced proliferation ability of TNBC cells and decreased the autophagy and apoptosis induced by flubendazole. Molecular docking was used to predict the binding mode of flubendazole with EVA1A, and the results suggested that flubendazole may interact with the Trp135, Thr113 and Asn110 residues of EVA1A. Subsequently, three site mutants of EVA1A ($EVA1A^{W135A}$, $EVA1A^{T113A}$ and $EVA1A^{N110A}$) were constructed and it was reported that only $EVA1A^{T113A}$ significantly weakened the inhibitory effect of flubendazole on MDA-MB-231 cells. This indicates that Thr113 may be a key amino acid residue involved in the binding of flubendazole to EVA1A and could serve an important role in regulating the proliferation and autophagy of TNBC cells. After treatment of breast cancer cells with flubendazole, the expression levels of DRP1 and PINK1 increased, promoting the mitochondrial translocation of Parkin to induce mitophagy by targeting EVA1A. Silencing EVA1A partially blocked DRP1 expression and Ser616 phosphorylation induced by flubendazole, reduced the colocalization of mitochondria and autophagosomes and decreased the expression of PINK1, Parkin and p-ParkinSer65. Conversely, overexpression of EVA1A in breast cancer cells can trigger DRP1-mediated mitophagy and significantly inhibit cell growth and proliferation (42).

p53. The p53 protein, encoded by the TP53 gene, is a crucial cancer suppressor. The functionality of p53 is complex, as it directly regulates the transcription of >300 target genes and indirectly affects the expression of thousands of genes. Its functions include cell cycle regulation, promotion of apoptosis, induction of cellular senescence, facilitation of DNA damage repair, maintenance of genomic stability, regulation of stem cell fate, suppression of metastasis, modulation of the balance between cell death and survival and regulation of glucose, lipid, amino acid, nucleotide, iron and redox metabolism (69).

Different melanoma cell lines exhibit distinct responses of p53 to flubendazole treatment. In A-375 cells, the p53

protein expression level significantly increased following treatment with flubendazole, while in BOWES cells, the p53 protein level remained stable. In RPMI-7951 cells, no p53 protein expression was detected in either the control group or the flubendazole-treated group, which is consistent with their known p53 null status (25). In CRPC, flubendazole can induce p53 protein expression. p53 increases p21/CDKN1A to inhibit the activity of Cyclin B1 and CDK1, thereby preventing cells from entering mitosis; it also transcriptionally represses SLC7A11 to reduce the uptake of cysteine within the cell, which in turn decreases the activity of GPX4 and triggers ferroptosis. Moreover, the activation of p53 may enhance the cytotoxicity of 5-FU, as the anticancer effect of 5-FU is partly dependent on the p53 signaling pathway (30). In GBM, flubendazole affects the p53 signaling pathway in a dose-dependent manner, upregulating the expression of the cancer suppressor proteins p53 and p21 and downregulating the expression of cyclin B1. The p53/p21/cyclin B1 signaling pathway induces cell cycle arrest in GBM cells, thereby promoting apoptosis (30).

PD-1. PD-1 is an immune checkpoint receptor expressed on the surface of various immune cells, including activated T cells, B cells, dendritic cells, monocytes, myeloid cells and natural killer cells. Within the cancer microenvironment, the interaction between PD-1 and its ligand, programmed death ligand 1 (PD-L1), is one of the critical mechanisms by which cancer cells undergo immune escape. The activation of PD-1 signaling can inhibit T cell receptor-mediated cytotoxicity and the proliferation of $CD8^+$ T cells, thereby preventing the clearance of cancer cells by the immune system. In cancer therapy, blockade of the PD-1/PD-L1 pathway can restore the ability of T cells to recognize and kill cancer cells, thus preventing immune escape (70,71).

In 2019, Li *et al* (51) reported that flubendazole could suppress the levels of PD-1 in human melanoma but had no effect on PD-L1 levels. Flubendazole inhibits the expression of PD-1 by suppressing STAT3, which may help enhance the immune system's response to cancer cells, thereby inhibiting cancer growth and metastasis. In 2023, Li *et al* (67) reported that flubendazole could reduce PD-1 protein expression levels in B16F10 melanoma cells from C57BL/6J mice without affecting PD-L1 levels. A reduction in PD-1 was accompanied by an increase in $CD3^+$ T cell infiltration, indicating that flubendazole may enhance the anticancer immune response by modulating PD-1 in the immune microenvironment. RNA-seq analysis of human Jurkat cells treated with flubendazole showed that flubendazole downregulated the mRNA expression level of PD-1 and upregulated the expression level of the transcription factor AP-1 family member Jun. When Jurkat T cells were pretreated with the AP-1 inhibitor T5224, the suppressive effect of flubendazole on PD-1 protein expression was blocked by T5224, indicating that the ability of flubendazole to inhibit PD-1 is, at least, partially dependent on the activation of AP-1.

JNK. JNK is a member of the MAPK family and is involved in a variety of cellular activities, including cell proliferation, differentiation, survival and death. The role of JNK in cancer is complex and multifaceted, and its specific function depends on a multitude of factors, such as the type of cancer cell and the microenvironment in which it resides. JNK can positively regulate autophagy, which can promote the survival of cancer cells under conditions of nutrient deficiency or stress, counteracting apoptosis. On the other hand, under conditions of sustained activation, JNK can activate proapoptotic proteins such as Bax and Bak, leading to cell apoptosis (72,73).

In melanoma cells treated with flubendazole, the activity of JNK gradually increased during the 24 to 48 h treatment period, peaking at 48 h in most cell types. The activation of JNK can lead to the phosphorylation of antiapoptotic proteins such as BCL-2, BCL-xL and MCL1 apoptosis regulator, which, once phosphorylated, undergo degradation or loss of function, ultimately promoting apoptosis. When melanoma cells were pretreated with the JNK-specific inhibitor SP600125 followed by the addition of flubendazole, the rate of apoptosis significantly decreased, confirming the important role of JNK in the apoptosis induced by flubendazole (31).

Autophagy-related protein 4B (Atg4B). Atg4B is a cysteine protease that is responsible for cleaving the C-terminal amino acids of Atg8 family proteins, including LC3 and gamma-aminobutyric acid A receptor-associated protein, thereby regulating the elongation and closure of autophagosomes. Additionally, Atg4B removes lipids from the Atg8 proteins that have fulfilled their function, thus promoting the maturation of autophagosomes, which is crucial for the proper formation and function of autophagosomes (74,75).

In TNBC, molecular docking and dynamics simulations suggested that flubendazole could form a stable complex with Atg4B. The binding of flubendazole to Atg4B leads to alterations in the activity of Atg4B, subsequently affecting the autophagic process. Furthermore, treatment with flubendazole significantly increased the production of reactive oxygen species (ROS) in MDA-MB-231 cells. ROS have been reported to regulate the substrate preference and activity of Atg4B, and flubendazole may facilitate autophagy by modulating ROS levels (76).

Hedgehog (Hh) signaling. The Hh signaling pathway is a conserved signaling pathway that serves a crucial role in embryonic development, the regulation of cell proliferation, differentiation, tissue regeneration and stem cell maintenance. This pathway is composed of core components, such as secreted proteins of the Hh family, including Sonic hedgehog (Shh), its receptors [Patched (Ptch) and Smoothened (Smo)] and the Gli family of transcription factors (Gli1, Gli2 and Gli3). In the absence of Hh signaling, Ptch inhibits the activity of Smo, thereby suppressing downstream signaling. When the Hh ligand binds to Ptch, the inhibition of Smo is lifted, activating downstream signals that ultimately regulate the expression of target genes through Gli transcription factors. Overactivation of the Hh signaling pathway can promote the proliferation, survival and dedifferentiation of cancer cells while also inhibiting apoptosis. Moreover, Hh signaling is associated with interactions with the cancer microenvironment, affecting cancer angiogenesis, invasion and metastasis (77,78).

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is an enzyme involved in cholesterol metabolism that is primarily responsible for regulating the levels of low-density lipoprotein cholesterol in the blood. High expression levels of PCSK9

in HCC tissues is correlated with a poor prognosis. PCSK9 can promote the carcinogenicity and metastasis of HCC by activating the Hh signaling pathway, particularly through the upregulation of Smo and Gli1 (35). Jin *et al* (35) revealed that treatment with flubendazole reduces the expression of the PCSK9 protein in HCC, increasing the uptake of cholesterol by HCC cells. This leads to the accumulation of cholesterol in the cell membrane, thereby inhibiting the activation of Smo and the activation of the Hh signaling pathway, resulting in the downregulation of Smo and Gli1 proteins. Furthermore, the combination of flubendazole with another multikinase inhibitor, lenvatinib, was more effective in treating HCC compared with lenvatinib alone, suggesting that flubendazole may affect the Hh signaling pathway by inhibiting PCSK9 and producing a synergistic effect with existing anticancer drugs (35).

4. Conclusions and future perspectives

The present review summarized that flubendazole can exert anticancer effects by inhibiting microtubule structure and function, inducing G2/M cell cycle arrest, inducing autophagy and apoptosis, suppressing cancer stem cell properties, inducing ferroptosis and inhibiting angiogenesis. Its anticancer mechanisms involve molecules and pathways such as the STAT3, NF-κB, AKT, EVA1A, p53, PD-1, JNK, Atg4B and Hh signaling pathways.

Further investigation is needed into the anticancer properties of flubendazole. First, the identification of additional cancer types sensitive to flubendazole is essential. Michaelis *et al* (14) screened a number of cancer cell lines with flubendazole and reported that the IC_{90} of flubendazole for 26 types of cancers was ≤5 μM. Future studies should consider the impact of flubendazole on a range of understudied cancers, including but not limited to Ewing sarcoma, head and neck cancer and medulloblastoma, cervical, ovarian, gastric, urothelial, renal cell and thyroid cancers (14). This research will enrich the current understanding of the anticancer effects of flubendazole and may reveal novel mechanisms of action. Second, enhancing the sophistication of experimental models is crucial for bolstering the reliability of findings. Notably, the majority of studies reported to date are based on cell line research, with a subset utilizing xenograft models. A limited number, specifically three studies, have employed primary patient cells for validation in patients with neuroblastoma (14), melanoma (31) and colorectal cancer (39). Therefore, future studies could benefit from employing patient-derived xenograft models (79), organoids (80,81) and conditionally reprogrammed cells (82,83). Third, the discovery of synergistic drug combinations with established chemotherapeutics, such as vinblastine, doxorubicin, paclitaxel, 5-FU and lenvatinib, in addition to the pleiotropic effects of flubendazole, is a compelling avenue for maximizing therapeutic efficacy and overcoming drug resistance (13,23,30,32,35,39,61,66). Fourth, due to its poor aqueous solubility and low oral absorption of flubendazole (1,5,84), addressing bioavailability challenges is essential. Innovative strategies such as high-oil-content nanoemulsions (85), novel nanocrystal formulation via microfluidization (86) and biphasic dissolution combined with the cylinder method have previously shown promise (87). Finally, the development of novel administration modalities, such as pulmonary delivery and intravesical chemotherapy, presents opportunities for the use of flubendazole in a targeted and effective manner. Compared with intravenous administration, the pulmonary route offers unique advantages for drug delivery due to the large surface area of the alveoli, thin epithelial barriers and rich blood supply, allowing for higher drug concentrations in the lungs (88). The exploration of inhalable formulations of flubendazole is an area of active research (89). Intravesical chemotherapy is a well-established treatment for superficial bladder urothelial carcinoma (90), and the application of flubendazole in this context could facilitate direct interaction with urothelial cancer cells. These avenues of research hold promise for advancing the therapeutic utility of flubendazole in cancer treatment.

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Authors' contributions

XX and ZZ were responsible for searching the literature and writing the original manuscript draft. HP and SC reviewed and edited the manuscript. SC and HP conceptualized the manuscript and approved the final version to be published. Data authentication is not applicable. All authors read and approved the final version of the manuscript.

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Competing interests

The authors declare that they have no competing interests.

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