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REVIEW

Withaferin A: A Dietary Supplement with Promising Potential as an Anti-Tumor Therapeutic for Cancer Treatment - Pharmacology and Mechanisms

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Abstract: Cancer, as the leading cause of death worldwide, poses a serious threat to human health, making the development of effective tumor treatments a significant challenge. Natural products continue to serve as crucial resources for drug discovery. Among them, Withaferin A (WA), the most active phytocompound extracted from the renowned dietary supplement *Withania somnifera* (L.) Dunal, exhibits remarkable anti-tumor efficacy. In this manuscript, we aim to comprehensively summarize the pharmacological characteristics of WA as a potential anti-tumor drug candidate, with the objective of contributing to its further development and the discovery of prospective drugs. Through an extensive review of literature from PubMed, Science Direct, and Web of Science, we have gathered substantial evidence showcasing WA's significant anti-tumor effects against a wide range of cancers in both in vitro and in vivo studies. Mechanistically, WA exerts its anti-tumor influence by inducing cell cycle arrest, apoptosis, autophagy, and ferroptosis. Additionally, it inhibits cell proliferation, cancer stem cells, tumor metastasis, and also suppresses epithelial-mesenchymal transition (EMT) and angiogenesis. Several studies have identified direct target proteins of WA, such as vimentin, Hsp90, annexin II and mFAM72A, while BCR-ABL, Mortalin (mtHsp70), Nrf2, and c-MYB are potential targets of WA. Notwithstanding its remarkable anti-tumor efficacy, there are some limitations associated with WA, including potential toxicity and poor oral bioavailability, which need to be addressed when considering it as an anti-tumor candidate agent. Nevertheless, I given its promising anti-tumor attributes, WA remains an encouraging candidate for future drug development. Unveiling the exact target and comprehensive mechanism of WA's action represents a crucial research direction to pursue in the future.

Keywords: Withaferin A, *Withania somnifera*, dietary supplement, anti-cancer activity, pharmacological mechanism, direct target

Introduction

Cancer poses a severe threat to human health and has emerged as a leading cause of death in many countries worldwide, particularly those experiencing rapid population growth and aging. According to the 2020 global cancer statistics, there were 19.3 million new cases and almost 10.0 million cancer-related deaths. It is projected that the number of cancer cases will rise to 28.4 million by the year 2040.[1] Consequently, there is an urgent need for novel anti-cancer drugs and treatment approaches.

Natural products have long been vital in drug discovery due to their unique biocompatibility, novel structural backbones, and diverse pharmacological activities. Many anti-cancer agents are either natural products or direct synthetic

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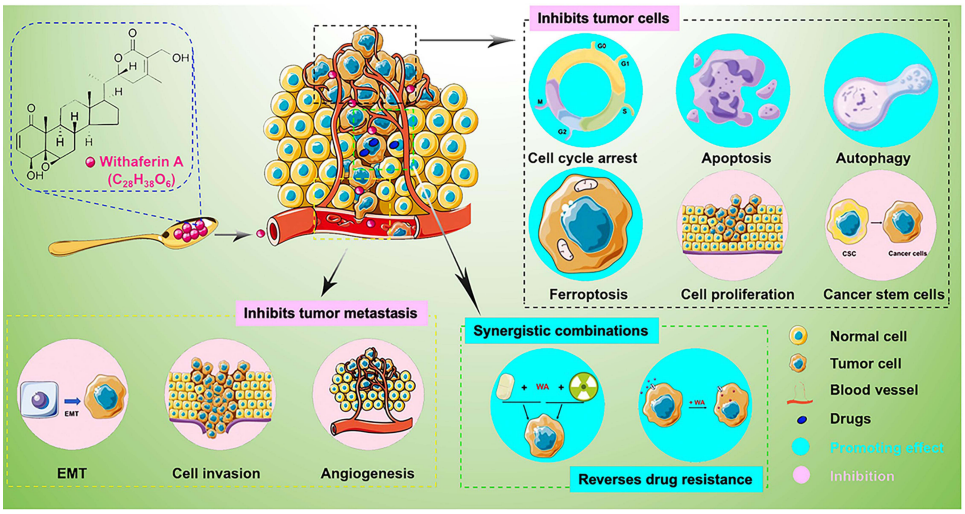
Graphical Abstract



derivatives of natural products,[2] such as paclitaxel, colchicine, irinotecan (a derivative of camptothecin). However, chemotherapeutic agents often come with undesirable side effects. As a result, dietary compounds derived from food sources are being explored as potential alternatives for anti-cancer drug discovery.

One such traditional medicine is *Withania somnifera* (L.) Dunal (WS), also kwon as Indian ginseng (*Ashwagandha*) and considered the king of Ayurvedic herbs, which has been utilized for 6, 000 years in Indian.[3] WS finds wide application in treating various conditions, including cancers, epilepsy, depression, arthritis, diabetes, Parkinson's disease, schizophrenia insomnia, and hypothyroidism, and palliative effects, such as analgesic, rejuvenating, regenerating, and growth-promoting effects, as well as improvement in sexual function.[4–9] Numerous research groups have investigated the chemical constituents of *Ashwagandha* to identify its bioactive entities. Withaferin A (WA) is a major bioactive lactone of WS (Figure 1). WA displays a wide range of activities, including anti-inflammatory, anticancer, anticoagulant, neuroprotective, hypoglycemic, hepatoprotective, and antiarthritic effects. Furthermore, recent study have reported that

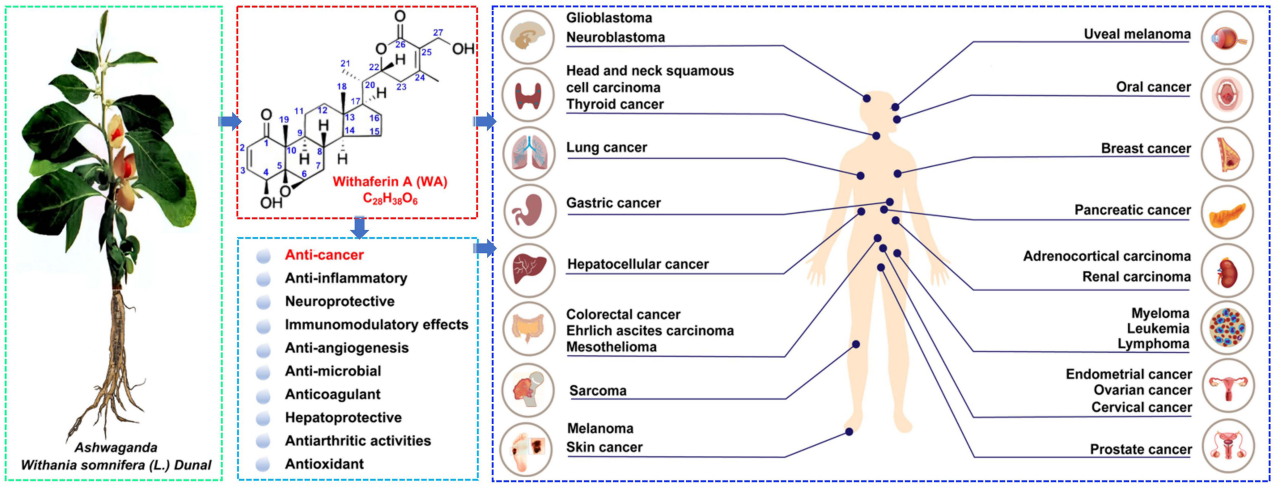


Figure 1 Role for different cancer types of WA isolated from *Withania somnifera*.

WA can potentially treat or prevent the COVID-19 transmission by inhibiting the virus's S protein from binding to the host receptor.[10]

Of particular note is WA's highly sensitive and broadly applicable anticancer efficacy. Studies have indicated that WA is a promising candidate and may become a potential treatment option for various cancers. In this review, we provide a comprehensive examination of the pharmacology and mechanism of WA as a potential drug candidate for cancer therapy. Additionally, we discuss viable strategies to overcome limitations and enhance the feasibility of WA as a novel anti-tumor agent.

The Anti-Tumor Efficacy of WA Against Various Cancers

Despite WA's popularity as a small molecule compound with diverse bioactivities, its antitumor activity has raised significant concerns. Nevertheless, WA shows promise in the treatment of multisystem tumors (Figure 1). In this study, we demonstrated the remarkable antitumor activity of WA in cancer cells (Table 1) and animal models (Table 2). Furthermore, we provided a comprehensive summary of the progress of WA in clinical trials.

In vitro Anti-Tumor Effects of WA

WA exhibits significant anti-tumor efficacy against almost all type cancers, Notably, it demonstrates promising results in reproductive system tumors, such as breast cancer,[11–13] cervical cancer,[33] ovarian cancer,[38,46] and endometrial cancer,[119] as well as urinary system tumors, including prostate cancer[48,49,125] and renal carcinoma.[59] Additionally, WA shows potential in combating digestive system tumors, such as oral cancer,[88,89,131,132] colorectal cancer (CRC),[61,62] pancreatic cancer,[68,69] hepatocellular carcinoma (HCC)[74,128] and gastric cancer,[118] as well as respiratory system tumors, including lung cancer.[76,77,141] Furthermore, it displays anti-tumor effects in endocrine system tumors like adrenocortical carcinoma,[116] and thyroid cancers,[87,130] circulatory system tumors like lymphoma[96] and leukemia[97,134] nervous system tumors including glioblastoma (GBM)[103] and neuroblastoma,[101] and motor system tumors like osteosarcoma.[109,110,142] Moreover, WA exhibits significant tumor inhibition in other types of tumors, including in uveal melanoma,[93] melanoma,[112] mesothelioma,[111] head and neck squamous carcinoma cells[117] and Ehrlich ascites.[138] The anti-tumor efficacy of WA is attributed to its ability to modulate multiple signaling pathways. It inhibits cell proliferation, migration, angiogenesis, and cancer stem cells (CSCs), while also inducing cell cycle arrest, apoptosis, autophagy, ferroptosis (Table 1).

In vivo Anti-Tumor Effects of WA

Various animal models have been utilized to evaluate the in vivo anti-tumor efficacy of WA (Table 2). The results consistently demonstrate that WA exhibits potent inhibitory effect on tumor growth and metastasis across multiple cancers when administered intraperitoneal (i.p.) or per os (p.o.) at doses ranging from 1–20 mg/kg. Moreover, WA has been showed to have synergistic effects when combinations with chemotherapeutics. In in vivo experiments, observations of mouse mortality and body weight suggest that WA is well-tolerated and safe, further indicating its potential for future development.

Clinical Research of WA

Given the significant antitumor activity of WA in cancers, several clinical trials have been conducted to investigate its safety and pharmacokinetics in the clinical treatment of cancer patients. Notably, a Phase I trial conducted by Pires N et al found that WA was generally well-tolerated in patients with advanced stage high-grade osteosarcoma at doses of 72, 108, 144 and 216 mg.[142] Furthermore, a recent clinical study titled "Combination therapy with liposomal doxorubicin and WA in recurrent ovarian cancer" aims to assess the feasibility and tolerance of WA in phase I and evaluate the treatment response (complete response (CR), partial response (PR), and stable disease (SD)) in recurrent ovarian cancer patients in Phase II (ClinicalTrials.gov Identifier: NCT05610735, 2022).

Table 1 Anti-Cancerous Activities of WA in vitro

Cancer	Cell Lines	Mechanisms of Cancer Cell Death	Refs
Breast cancer	MCF-7, MDA-MB-231, SUM159, SK-BR-3, BT474, ERBB2, T47-D, MDA-MB-468, MCF-10A, BT-20, SUM149, 231MFP, MDA-MB-361, 231MFP	Inducing apoptosis, G2/M arrest, autophagy. Inhibition of EMT, stemness. Inhibition of NF-κB, STAT3, Notch1/2/4, ERα signaling. Activation of ERK/RSK. Alteration of MAPK signaling.	[11–32]
Cervical cancer	Caski, SK-Hep1, HeLa, SKGII, SKGIIIb, and ME180	Inducing apoptosis, G2/M arrest. Repression of HPV oncogenes.	[33–37]
Ovarian cancer	A2780, SKOV3, A2780 OKV18, CAOV3, A2780/CP70	Inducing autophagy, apoptosis. Targeting putative cancer stem cells. Generation of ROS. Activation of p53. Down-regulation of Notch1, Notch3.	[33,38–47]
Prostate cancer	LNCaP, PC3, 22RV1, DU145	Inducing autophagy, oxidative stress, mitotic catastrophe, G2/M arrest, FOXO3a-dependent apoptosis. Inhibition of AKT signaling.	[48–58]
Renal carcinoma	Caki	Inducing endoplasmic reticulum stress (ERS) mediates apoptosis.	[59,60]
Colorectal cancer	WS480, HT-29, HCT-116, RKO, SW-480, SW-620, Caco-2	Inducing ERS-mediated autophagy and apoptosis, G2/M arrest. Inhibiting of STAT3, Notch-1 signaling. Downregulating of AKT/NF-κB /Bcl-2.	[61–67]
Pancreatic cancer	PANC-28, BxPC-3, MIA PaCa-2, AsPC-1, Panc-1, SW1990	Inhibition of proteasome. Inducing ERS-mediated apoptosis, autophagy. Targeting pancreatic CSCs, heat shock protein 90. Inactivating of PI3K/Akt pathway.	[61,68–73]
Hepatocellular cancer	Huh7, HepG2, MHCC97H and MHCC97L	Inducing autophagy and apoptosis.	[74,75]
Lung cancer	H1355, PC9, H358, H928, CL1-0 CL1-1, CL1-3, CL1-5, H157, H520, A549, PC13, PC14, H1299, H460, CL1-0, CL1-1, CL1-3, CL1-5, PC13 and PC14	Inducing autophagy, apoptosis. Activation of ROS. Inhibition of cell adhesion, migration, invasion, the growth of lung CSCs. Inhibition of mTOR/STAT3 signaling, AK4-HIF-1α signaling axis.	[76–86]
Thyroid cancer	SW1736	Inducing apoptosis.	[87]
Oral cancer	Ca9-22, CAL 27, HSC-3, SCC-4, HSC-4	Inducing apoptosis and DNA damage, G2/M arrest, autophagic cell death, endogenous ROS production. Inhibition of ROS-mediated migration, invasion, targeting MAPK/RAS/RAF signalling pathway.	[88–92]
Uveal melanoma	OMM2.3, 92.1, and MEL290	Inducing G2/M arrest and apoptosis. Suppression of c-Met, AKT, and Raf-1 signaling.	[93]
Skin cancer	JB6 Cl-41 P+	Suppressing of up-regulation of acetyl-coA carboxylase 1, ubiquitin-proteasome pathway, isocitrate dehydrogenase 1 activity and mitochondrial function.	[94,95]
Hematologic tumor	LY-3, LY-10, SudHL-6, 37, HL-60, MDS-L, THP-1, Jurkat and Ramos	Inducing cell cycle arrest, apoptosis, autography. Inhibition of NF-κB nuclear translocation.	[96–100]
Neuroblastoma	Be(2)-c, SMS-KCNR, SH-SY5Y, LAN-5, IMR-32, IMR-32, SK-N-SH, Kelly, NB69, CHP-134, IMR-32, NLF, CHP-212, SK-N-DZ, SK-N-BE(2)-C, SK-N-AS, SH-EP	Inducing ferroptosis. Inhibition of STAT3.	[101,102]

(*Continued*)

Table 1 (Continued).

Cancer	Cell Lines	Mechanisms of Cancer Cell Death	Refs
Glioblastoma	U87, U251, GL26, YKG1, U118MG, A172	Inducing G2/M arrest, intrinsic apoptosis. Combined inhibition of NF-κB and STAT3 activation.	[103–108]
Sarcoma	S-180, U2OS	Inducing apoptosis, generation of ROS and disruption of mitochondrial membrane potential.	[109,110]
Mesothelioma	H2373, H2452, H2461, H2595, H226 MPM	Inducing apoptosis. Decreasing the chymotryptic activity of the proteasome	[111]
Melanoma	MelJD, MelCV and MM200, M14, Mel501, SK28, Lu1205, WM793	Inducing apoptosis. Generation of ROS. Down-regulation of Bcl-2.	[85,112–115]
Adrenocortical carcinomas	Y1, SW13	Inducing cell cycle arrest from G1/G0 to G2/M, apoptosis. Modulating expression of Jagged 1, MAPK, and AKT/mTOR pathway proteins.	[116]
Head and neck squamous cell carcinoma	MDA1986, JMAR, UM-SCC-2, and JHU011	Inducing apoptosis, cell death, cell-cycle shift from G(0)/G(1) to G(2)/M.	[117]
Gastric cancer	AGS	Inducing G2/M arrest and apoptosis.	[118]
Endometrial cancer	KLE	Inducing G2/M arrest and apoptosis.	[119]

Table 2 Anti-Cancerous Activities of WA in vivo

Cancer	Animal Model	Administration Dose and Method	HED (mg/kg)	Refs
Breast cancer	MDA-MB-231 xenograft in nude mice	i.p. 8 mg/kg for 2.5 weeks	0.65 mg/kg	[13]
		p.o. 5mg/kg 5 days a week for 4 weeks	0.41mg/kg	[20]
		i.p. 5–20 mg/kg 3 times a week for 6 weeks	0.41–1.63mg/kg	[120]
		i.p. 100 μg 5 days a week for 7.5 weeks	8.13μg	[121]
		i.p. 4mg /kg 5 days a week	0.33mg/kg	[28]
	4T1 mouse mammary carcinoma model	i.p. 4 mg/kg every other day for 1 month	0.33mg/kg	[19]
		i.p. 5–10 mg/kg every other day for 2 weeks	0.41–0.81mg/kg	[122]
		i.p. 1,4,8mg/kg 3 times a week for 4 weeks	0.08, 0.33, 0.65mg/kg	[123]
	N-methyl-N-nitrosourea (MNU)-rat	4–8 mg/kg	0.33–0.65mg/kg	[124]
Cervical cancer	CaSki xenograft in nude mice	i.p. 8 mg/kg on alternate days for 6 weeks	0.65mg/kg	[34]

(*Continued*)

Table 2 (Continued).

Cancer	Animal Model	Administration Dose and Method	HED (mg/kg)	Refs
Ovarian cancer	A2780 xenograft in nude mice	i.p. 2 mg/kg every other day for 12 days i.p. 2 mg/kg every other day for 4 weeks	0.16mg/kg 0.16mg/kg	[45] [38]
	A2780 xenograft in NOD.Cg mice	i.p. every 3 days at 2 mg/kg or 6 mg/kg for 5 weeks	0.16mg/kg	[42]
	A270 intraperitoneal tumors in nude mice	i.p. 2 mg/kg every third day for 3 weeks	0.16mg/kg	[44]
Prostate cancer	PC-3 xenograft in nude mice	Intratumor injection 5 mg/kg 5 days a week for up to 4 weeks i.p. 4–8 mg/kg for 7 days	0.41mg/kg 0.33–0.65mg/kg	[56] [125]
	Transgenic Hi-Myc mice	i.p. 0.1mg/mouse 3 times/week for 5 weeks	0.41mg/kg	[55]
	Ptenloxp/loxp: PB-Cre4 (Pten-KO) Mice	p.o. 3 and 5 mg/kg for 45 weeks	0.24 and 0.41 mg/kg	[49]
	C57BL/6-Tg [TRAMP] 8247Ng/J	p.o. 3 and 5 mg/kg for 39 weeks	0.24 and 0.41 mg/kg	[126]
	pCMV/DU-145 or AKT/DU-145 xenograft in Balb/c mice	p.o. 4mg /kg for 4 weeks	0.33mg/kg	[57]
Colorectal cancer	HCT-116 xenograft in nude mice	i.p. 2 mg/kg every 2 days for 32 days p.o. 5 mg/kg 5 days per week for 4 weeks.	0.16mg/kg 0.41mg/kg	[62] [63]
	C57BL/6 and $APC^{Min/+}$ mice azoxymethane/ dextran sodium sulfate (AOM/DSS) model	p.o. 4mg/kg 5 days/week for 12 weeks p.o. 3mg/kg 5 days/week for 10 weeks	0.33mg/kg 0.24mg/kg	[127]
Pancreatic cancer	Panc-1 xenografts in nude mice	i.p. 3 mg/kg every other day for 7 weeks i.p. 3–6 mg/kg 2 times a week for 4 weeks i.p. 3–6 mg/kg every other day for 45 days	0.24mg/kg 0.24–0.49mg/kg 0.24–0.49mg/kg	[73] [69] [68]
Hepatocellular cancer	HepG2-xenografts and DEN-induced-HCC in C57BL/6 mice	4 mg/kg daily for 5 weeks	0.33mg/kg	[128]
Lung cancer	H441-L2G xenografts in NOD/SCID mice	2 mg/kg for 6 weeks	0.16mg/kg	[76]
	PC9 xenografts in BALB/c nude mice	2 mg/kg 3 times per week	0.16mg/kg	[129]
	CL1-0 AK4 and A549-GL cells xenografts in NOD-SCID Gamma mice	i.p. 4 mg/kg 3 times a week for 4 weeks	0.33mg/kg	[79]
Thyroid cancer	DRO 81–1 xenografts in nude mice	i.p. 8 mg/kg every day for 21 days	0.65mg/kg	[130]

(*Continued*)

Table 2 (Continued).

Cancer	Animal Model	Administration Dose and Method	HED (mg/kg)	Refs
Oral cancer	HSC-4 xenografts in NOD-SCID mice	i.p. 2 mg/kg 3 times per week for 13 weeks	0.65mg/kg	[88]
	DMBA-induced hamster buccal pouch carcinogenesis/ oral squamous cell carcinoma	p.o. 20 mg/kg	1.63mg/kg	[89,131,132]
Uveal melanoma	92.1 xenograft in nude mice	i.p. 8–12 mg/kg every day for 21 days	0.65–0.98mg/kg	[93]
Skin cancer	TPA skin cancer model	Topical application of 20 mg 5 times per week for 14 weeks	1.63mg/kg	[133]
Hematologic tumor	A20 allograft in Balb/c mice Notch1-mutant T-ALL xenograft in NRG mice	12 mg/kg every other day for 2 weeks 10mg/kg	0.98mg/kg	[96] [134]
Neuroblastoma	IMR-32 xenograft in nude mice	Intratumor injection 4 mg/kg daily for up to 20 days	0.33mg/kg	[101]
Glioblastoma	U87 xenograft in nude mice	Injection 5 mg/kg into the tail vein every day for 27 days	0.41mg/kg	[103]
Sarcoma	Sarcoma xenograft in Swiss albino mice	i.p. 10 mg/kg for 7 days	0.81mg/kg	[135]
	S-180 xenograft in Swiss or DBA/2 mice	10 or 30 mg/kg	0.81 or 2.44 mg/kg	[109]
Mesothelioma	AB12 xenograft in Balb/c mice.	i.p. 5 mg/kg for 17 days	0.41mg/kg	[111]
Melanoma	B16F1 melanoma xenograft in C57BL mice	i.p. 15 mg/kg daily, 5 days a week for 3 weeks. i.p. 2.5 mg/kg every other day i.p. 40 mg/kg	1.22mg/kg 0.20mg/kg 0.33mg/kg	[114] [136] [137]
Ehrlich ascites carcinoma	Xenograft in Inbred Swiss albino mice	i.p. 5–30 mg/kg i.p. 10–60 mg/kg Injection of 25–60 mg/kg	0.41–2.44mg/kg 0.81–4.88mg/kg 2.03–4.88mg/kg	[138] [139] [140]

Abbreviation: HED, human equivalent dose.

The Reported Direct Binding Target of WA in Cancers

Identifying the specific targets of compounds plays a crucial role in elucidating their mechanism of action and in drug development. Active natural compounds often act on multiple targets. Several studies have identified candidate targets of WA through computer calculations and molecular simulations (Table 3). BCR-ABL,[143] ACE2,[144] Mortalin (mtHsp70) and Nrf2[145] were proposed as potential targets of WA based on these approaches. Furthermore, Clesham et al utilized the Connectivity Map (CMAP) database and identified c-MYB as a potential target of WA in acute myeloid leukemia.[97]

Additionally, a few articles have demonstrated that vimentin, Hsp90, and annexin II proteins directly bind to WA using WA-biotin analogs (Table 3). Paola BM et al revealed a covalent bonding between the C3 or C6 electrophilic carbon centers of WA and Cys328 of the vimentin A-helix. Moreover, hydrogen bonding was observed between the C1 position oxygen atom and Gln324 of the vimentin A-helix, as well as between the C4 hydroxyl group and Asp331 of the vimentin A'-helix.[146] Yanke et al identified that WA-biotin binds to the C-terminus of Hsp90.[69] Additionally, both Falsey RR et al and Gabriel Ozorowski et al demonstrated that WA directly binds to annexin II protein.[147,148] However, Falsey RR et al found that WA binds covalently to the N-terminal domain of annexin A2, not Cys133. Furthermore, Jessica et al reported WA binds to mFAM72A with micromolar affinity in HeLa and HEK293T cells using biolayer interferometry.[149]

Table 3 The Direct Target Proteins of WA in Cancers

Compound	Experimental Methods	Target Proteins	Binding sites and Mechanism	Refs
WA	Molecular docking Molecular dynamics simulations	BCR-ABL	Interacts at both catalytic and allosteric sites of the ABL. Acts as both catalytic and allosteric inhibitor of the ABL.	[143]
		ACE2	Significantly inhibits the ACE2 expression.	[144]
		Mortalin (mtHsp70)	The amino acids directly interacting with WA are GLU448, LEU450, GLN479, THR449, PHE472, GLU483, ALA475. Inhibition of Mortalin (mtHsp70).	[145]
		Nrf2	The amino acids directly interacting with WA are ILE559, VAL420, VAL606, VAL467, CYS368, THR560, CYS513. Inhibition of Nrf2 protein.	[145]
	Screening CMAP database	c-MYB	Induces rapid ablation of c-MYB protein and consequent inhibition of c-MYB target gene expression	[97]
	Using WA-biotin analogs	Vimentin	Binds to the vimentin by covalently modifying its cysteine residue, which is present in the highly conserved α-helical coiled coil 2B domain. Covalent bonding between Cys328 of the vimentin A-helix and the C3 or C6 electrophilic carbon centers of WA. Hydrogen bonding between Gln324 of the vimentin A-helix and the C1 position oxygen atom, and Asp331 of the vimentin A′-helix and the C4 hydroxyl group. Causes aggregation of vimentin filaments.	[146]
		Hsp90	Binds to C-terminus of Hsp90. Inhibits Hsp90 chaperone activity through an ATP independent mechanism.	[69]
		Annexin II	A covalent bond between Cys133 of annexin II and C3 or C5 of WA. Disrupts the actin cytoskeleton in an annexin II–dependent manner.	[147]
	Using mass spectrometry and single-site mutants	Annexin II (AnxA2)	WA-AnxA2 interaction to the N-terminal domain of AnxA2 where WA binds covalently to Cys9.	[148]
	Using biolayer interferometry	mFAM72A	Binds mFAM72A with micromolar affinity. To be a FAM72A inhibitors	[149]

Molecular Mechanism of the Antitumor Effect of WA in Various Cancers

The complete understanding of the mechanisms underlying WA's antitumor activity remains elusive; nevertheless, it appears to involve multiple effects, such as inducting cell cycle arrest, apoptosis, autophagy, ferroptosis, and suppressing invasion, metastasis, angiogenesis, and cancer stem cells.[150–152] In light of this, we conducted a comprehensive review of the molecular mechanism of the antitumor activity of WA in various cancers (Figure 2).

Inducing Cell Cycle Arrest

In various cancers, including human breast cancer cells, ovarian cancer cells, cervical cancer cells,[34,46] uveal melanoma cells,[93] human head and neck cancer cells,[117] and GBM cells,[104,105] WA has been reported to induce G2/M phase arrest. The process of WA-induced G2/M arrest involves multiple cell cycle-related proteins, such as cyclin-dependent kinase 1 (Cdk1), cyclin B1, cell division cycle 25C (Cdc25C) and Cdc25B. In MCF-7 and MDA-MB-231 cells, WA treatment led to a concentration-dependent and time-dependent decrease in the expression levels of Cdk1, Cdc25C and Cdc25B, ultimately inducing G2/M arrest.[153] Moreover, overexpression of Cdc25C in breast cancer cells partially prevented the G2/M arrest induced by WA.[153] Similarly, WA treatment downregulated Cdc25C and induced cyclin B1 in GBM and ovarian cancer cells CaOV3 and SKOV3.[34,105] Consistent with Stan's findings,[153] WA-treated CaSki cells showed an accumulation of cyclin B1, downregulation of Cdk1, decreased complex formation between cyclin B1 and Cdk1,[34] and induction of the Cdk inhibitor p21.[125] Additionally, clues for G2/M arrest induced by WA emerged from the observation of mitotic spindle disruption microtubules.[109]

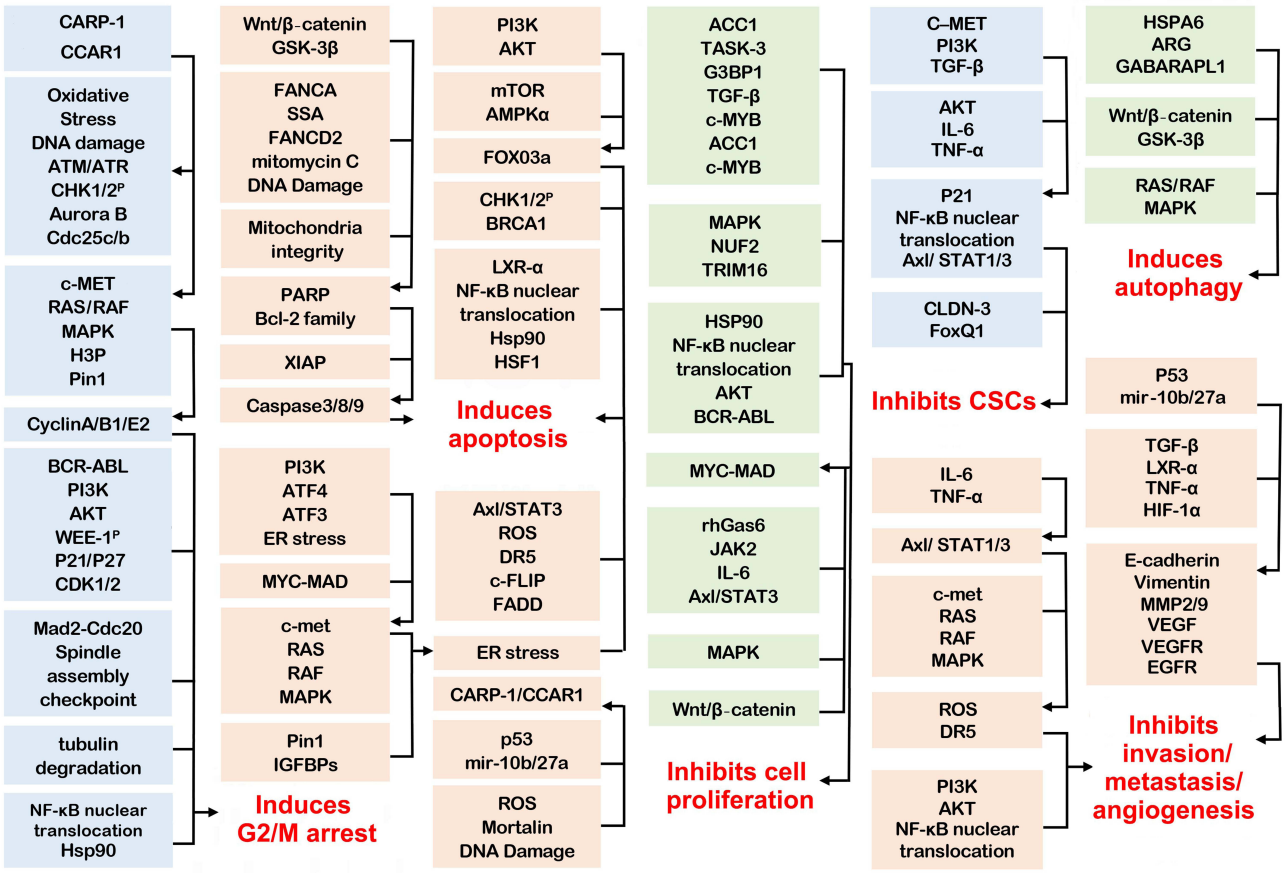


Figure 2 Mechanisms and signaling pathway of WA on cancer inhibition.

Inducing Apoptosis

Apoptosis, a form of programmed cell death, is a common mechanism employed by antitumor drugs. In human cells, there are two well-characterized pathways that trigger apoptosis. One is the intrinsic pathway, which is induced by mitochondrial changes. The activation of caspases is regulated by mitochondrial Bcl-2 family proteins.[154,155] Another is the extrinsic pathway, activated by death domain-containing receptors, such as CD95, TNF receptors and TNF-related apoptosis-inducing ligands.

Inducing apoptosis is the primary anti-tumor effect of WA, observed in breast cancer,[13] prostate cancer, leukemia,[156] melanoma,[113] and head and neck cancer. WA induces the generation of the reactive oxygen species (ROS) in many cancer cells, leading to increased expression of Bak and Bax, which in turn induces mitochondrial apoptosis. Additionally, WA triggers Bak and Bax protein activation by reducing laminin and integrin gene expression.[14] In CRC cells, WA induces apoptosis through ROS-mediated mitochondrial dysfunction and the JNK pathway.[61] Tang's study revealed that WA triggered intrinsic apoptosis in GBM cells via the ATF4-ATF3-CHOP axis.[103] Another study demonstrated that WA induced apoptosis by inhibiting the AKT/mTOR pathway. Silvia's research indicated that by knocking down Bim and FOXO3a levels in breast cancer cell lines, WA-induced apoptosis was significantly attenuated in vivo.[13]

Moreover, it has been documented that WA enhances TNFα-related apoptosis-inducing ligand-induced apoptosis (TRAIL) by decreasing the expression of c-FLIPL and c-FLIPs (cellular FLICE-like inhibitory protein), the negative regulators of apoptosis.[157–160] In Seon's study, WA induced cell apoptosis by inhibiting the phosphorylation of Axl and STAT3 induced by the growth inhibition specific protein 6 (rhGas6).[161] The inhibition of STAT3 (on Tyr705) phosphorylation also results in inhibition of Janus-activated kinase 2 (JAK2) activity. Furthermore, WA causes apoptosis by down-regulating STAT3-regulated genes, including Bcl-2, Bcl-xL, survivin and cyclin D1, in human renal carcinoma Caki cells.[59]

Inhibiting Proliferation

Additionally, WA has demonstrated the ability to inhibit tumor cell proliferation through various mechanisms. For instance, in human endometrial carcinoma, WA inhibits tumor cell proliferation by blocking the phosphorylation of TGF-β-dependent Smad2 and the expression of other TGF-β-related proteins.[119] In the case of myeloma cells, WA inhibits proliferation through ROS-mediated intrinsic apoptosis.[162] Moreover, WA has been reported to inhibit proliferation in chronic myeloid leukemia by targeting BCR-ABL oncogenic signaling.[143] Furthermore, WA exerts its inhibitory effects on proliferation by regulating c-MYB target gene expression. This occurs through the induction of c-MYB protein ablation, resulting in reduced viability, impaired colony formation, and hindered progression of acute myeloid leukemia cells.[97] Additionally, combined with blocking SGs by targeting G3BP1, WA-induced oxidative stress combined with blocking SGs by targeting G3BP1 results in reduced survival of prostate cancer.[50]

Inducing Autophagy

Physiologically, autophagy is a cellular process responsible for degrading macromolecules and organelles and has been shown to contribute to cell death.[163,164] Inducing autophagy is an important anti-tumor mechanism of WA. In three WA-treated prostate cancer cell lines (22Rv1, PC-3 and LNCaP), autophagy induction was confirmed through the results of transmission electron microscopy and Western blot analysis. The key autophagy markers, including LC3BII, SQSTM1, Atg7 and Beclin-1, were robustly increased in WA-induced cancer cells. Moreover, GABARAPL1 was found to be involved in the cytoprotective autophagy induced by WA in prostate cancer cells.[48]

Additionally, WA inhibits the expression of β-catenin and p-GSK-3β proteins, leading to autophagy.[165] Recent studies have shown that WA induces autophagy in both a spontaneously immortalized and nontumorigenic normal human mammary epithelial cell line (MCF-10A) and human breast cancer cells (MCF-7 and MDA-MB-231). Furthermore, WA induces autophagy in MDA-MB-231 xenograft mice.[15]

Inducing Ferroptosis

Ferroptosis is characterized by uncontrolled lipid peroxidation of cell membranes, resulting in membrane damage and cell death.[166,167] Recent studies have reported that WA can induce a form of nonapoptotic cell death known as ferroptosis in cancer cells. This induction is attributed to the excessive activation of heme oxygenase-1 (HMOX1) by WA, which elevates intracellular labile ferrous iron (Fe^{2+}) levels, consequently leading to the accumulation of toxic lipid radicals and triggering ferroptosis.[101] Additionally, WA has been found to induce ferroptosis through directly targeting and inhibiting glutathione peroxidase 4 (GPX4), which plays a critical role in detoxifying membrane hydrogen peroxide.[168] Notably, according to Emilie, due to considerable overlap in ferroptosis and apoptosis kinome activity, WA induces mixed ferroptosis and apoptosis in multiple myeloma cells.[169]

Suppressing Cancer Stem Cells

Cancer stem cells (CSCs) are a subpopulation of cells within tumors that possess self-renewal and differentiation capabilities, and they are believed to play a significant role in tumor initiation, growth, metastasis, and therapy resistance.[39] WA has demonstrated properties in suppressing CSC as well. Kakar et al reported the role of WA in suppressing putative CSCs in ovarian cancer.[38] They observed a remarkable 70–80% reduction in tumor metastasis and growth of cancerous cells. WA significantly inhibited the expressing of CSC markers, including CD24, CD44, CD117, CD34 and Oct 4, and downregulated the Hey 1, Notch 1, and Hes 1 genes. Similarly, Jade et al demonstrated that WA effectively inhibits the growth of lung CSCs, the formation of lung cancer spheroids, and decreases side population cells.[76] Additionally, Kim and Singh reported that FoxQ1 is a target of WA for inhibiting breast CSCs in vivo.[11] Moreover, Mayuko et al found that WA is a potent inhibitor of CSC stemness, leading to cellular senescence primarily via the induction of p21Cip1 expression.[170] Therefore, WA holds promise for providing potential therapeutic benefit in various cancers by suppressing CSCs through diverse pathways, making it a promising candidate for further investigation as a potential therapeutic agent for different types of cancer.

Inhibiting Cancer Metastasis and Angiogenesis

Epithelial mesenchymal transition (EMT) is a biological process in which cells lose their epithelial characteristics and acquire mesenchymal properties, facilitating the migration and invasion of tumor cells. Studies have demonstrated that WA decreases cellular mobility by countering EMT, such as decreasing Slug (SNAI2) and Twist expression, while increasing the adhesion molecule E-cadherin expression.[63,170,171] In human non-small cell lung cancer (NSCLC) cells, WA suppresses TGFβ1- and TNFα-induced EMT and inhibited cell adhesion, invasion and migration of H1299 and A549 cells. Moreover, WA inhibits EMT by preventing the nuclear translocation and phosphorylation of Smad2/3 and nuclear factor kappa B (NF-κB) in H1299 and A549 cells.[77] Additionally, Chen et al reported that WA inhibits the migration of lung cancer cells by downregulating miR-27a and miR-10b, which regulate the expression of Bax and E-cadherin.[78] Furthermore, WA suppresses the AK4-HIF-1α signaling axis and acts as a potent antimetastatic agent in lung cancer.[79] In MMTV-neu mouse and breast cancer xenografts, WA increases E-cadherin expression and reduces vimentin expression.[16] Moreover, at a concentration of 700 nM, WA suppresses breast cancer metastasis and relapse by inhibiting the urokinase-like plasminogen activator (uPA) protease, which promotes cell migration and proliferation by remodeling the extracellular matrix.[17,18] Another study demonstrated that 3-azido-WA upregulates TIMP-1 and E-cadherin expression in prostate cancer cells.[135] As a vimentin inhibitor, WA suppresses the migration and invasion activity of glioblastoma cells by inhibiting vimentin.[172] Additionally, WA interacts with vimentin and heterogeneous nuclear ribonucleoprotein hnRNP-K to downregulate the expression of proteins involved in tumor cell metastasis, such as MMPs, VEGF, N-cadherin, and u-PA.[173]

Furthermore, WA exerts potent anti-angiogenic activity in vivo.[174] In the Ehrlich ascites tumor model, WA exerts its anti-angiogenic activity by reducing the binding of the transcription factor specificity protein 1 (Sp1) to VEGF.[175] S Saha et al demonstrated that WA has a favorable binding affinity with vascular endothelial growth factor (VEGF), leading to decreased angiogenesis.[176] In another study, WA reduces macrophage infiltration and inhibits the expression of protein tyrosine kinase-2 (Pyk2), rho-associated kinase 1 (ROCK1), and VEGF in a hepatocellular carcinoma xenograft model, thereby suppressing tumor invasion and angiogenesis.[177] WA can also directly interact with the hnRNP residue domain through hydrogen bonding and hydrophobic interactions, disrupting the binding between RNA-binding protein hnRNP-k (hnRNP-k) and single-stranded DNA (ssDNA). This inhibits hnRNP-k from binding to ssDNA and subsequently lowers the downstream effects of hnRNP-k, including the expression of VEGF, PERK, and MMP2, thus suppressing the migration and invasion of HT1080 fibrosarcoma cells.[178] Moreover, a recent study found that WA treatment downregulates the secretion of many angiogenesis proteins in HCC.[74] According to Bilal's findings, 3-azido WA dose-dependently suppresses the expression of p-ERK and p-AKT, which may play a significant role in inhibiting angiogenesis in mouse.[179] By reducing Akt signaling and MMP-9 expression, WA also decreases the invasion and migration capabilities of CasKi cells.[35]

Synergistic Combinations

Since the first approval of synergistic combination drugs in the 1940s, the number of approved synergistic combination has experienced significant growth. The ideal synergistic combination can improve clinical efficacy, reduce drug toxicity, and delay or prevent the development of drug resistance. As a highly effective antitumor agent, WA has been studied in combination with several other drugs.

In one combination of WA and cisplatin, WA produced ROS, while cisplatin caused DNA damage, suggesting that lower doses of cisplatin combined with suboptimal doses of WA could achieve the same effect.[40] Kendra's study demonstrated the combinatorial of sulforaphane and WA showed synergistic effects on epigenetic modifiers and cell proliferation in breast cancer cells.[180] Cohen et al reported that a synergistic combination of WA and sorafenib caused G2/M arrest in anaplastic and papillary thyroid cancer cells.[87] Abdullah et al reported that the combination of WA and 5-FU executed PERK axis-mediated endoplasmic reticulum (ER) stress-induced autophagy and apoptosis by upregulating the expression of ER stress sensors, such as PERK, BiP, CHOP, eIF2α, and ATF-4.[64] The combination also modulated ER stress and significantly induced antiproliferative effect and cell death in CRC cells.[64] Additionally, the combination of cisplatin and pemetrexed with WA synergistically inhibited wild-type epidermal growth factor receptor

(EGFR) lung cancer cell viability. Moreover, WA further enhanced the cytotoxic effect of cisplatin in lung CSCs.[76] The combination of WA with tumor treating fields (TTFields) showed obvious synergistic effects by significantly inhibiting tumor growth in glioma cells (GBM2, GBM39, U87-MG).[106] WA and carnosol also exhibit a synergistic effect on pancreatic cancer cells through targeting pancreatic cancer stem cells by phosphorylating c-met and downregulating pluripotency maintenance genes (Oct-4 and Nanog).[70]

Reversing Drug Resistance

Over the past two decades, drug development in the field of oncology has predominantly focused on molecular targeted drugs. However, the rapid emergence of drug resistance due to target mutations has significantly reduced drug efficacy, making overcoming drug resistance a major challenge for current antitumor drugs. Multiple studies have found that WA has the ability to reverse drug resistance in various cancers.

Kunimasa et al demonstrated that the combination of WA and phloretin (a glucose transport inhibitor) led to a significant reduction in tumor size in gefitinib-induced drug- tolerant lung cancer, indicating that EGFR-resistant lung cancer could be effectively treated with a combination of WA and metabolism-targeting therapies.[129] In sorafenib-resistant HCC cells (HepG2 and SNU449 cells), WA enhanced ferroptosis and increased Keap1 expression to counteract the effects of Nrf2 signaling activation on the ferroptosis-related protein xCT and EMT. Moreover, blockade of Keap1/Nrf2 signaling facilitated sorafenib resistance and reversed WA-induced ferroptosis. Consequently, WA attenuated sorafenib resistance and metastatic potential by regulating Keap1/Nrf2-associated ferroptosis and EMT.[181] In sorafenib-resistant HepG2 cells, WA induced a dose-dependent reduction in vimentin expression, followed by a reduction in ABCG2 expression and a decrease in cell viability, induced by the inhibition of vimentin in both parental and sorafenib-resistant HepG2 cells.[182] WA also repressed the invasiveness of cyclophosphamide, vincristine, doxorubicin, and prednisone (CHOP) chemotherapeutic regimen-resistant DLBCL cells in collagen matrices.[183] In GBM, WA could resensitize temozolomide-resistant GBM cells by depleting O6-methylguanine-DNA methyltransferase (MGMT) and inducing apoptosis through the AKT/mTOR pathway.[107]

Limitations of WA as a Potential Anti-Cancer Candidate and Corresponding Solutions

Despite the broad antitumor effects of WA, there were also some limitations, including potential toxicity, poor oral bioavailability, and low production.

Potential Toxicity

The toxicity of natural active compounds is often unavoidable, and WA is no exception. However, the toxicity of WA has been a subject of controversial and potential concern. As the main bioactive component of WS, there have been articles demonstrating the safety of WS extract in all tested groups.[184,185] An acute and sub-acute toxicity study of oral WA also yielded similar results, showing that the LD_{50} of WA in mice was above 2000 mg/kg body weight.[186] On the other hand, other studies found that WA exhibited certain toxic side effects in mice, with an LD_{50} of 54 mg/kg body weight.[187,188] To address these toxicity concerns, researchers have explored structural modifications to obtain derivatives of WA with comparable but safer activity. For instance, the 2-thiophene ester-linked derivative of WA, ASR488, selectively inhibited bladder cancer cells while showing no toxicity to normal cells.[189] Furthermore, a range of IC_{50} values for withanolides, as reported by Zhang et al, suggests the existence of various compounds within this class with potential anti-cancer activity, warranting further exploration and development.[190] Notably, the ability of another WA derivate, ASR490, to inhibit the growth of colon cancer cell lines and xenotransplanted tumors without causing systemic toxicity is encouraging.[191]

Overall, these studies highlight the potential of WA as a source of lead compounds for the development of new anti-cancer drugs. However, further research in this area is essential to fully understand and address the potential toxicity concerns associated with WA and its derivatives.

Poor Oral Bioavailability

In addition to the potential toxicity issues, another limitation of WA is its poor oral bioavailability. Saurabh by Saurabh et al and Tianming et al reported oral bioavailability values 1.8% and 32.4 ± 4.8%, respectively, in male rats.[186,192] The poor oral bioavailability of WA limits its effectiveness as a drug for cancer prevention and treatment.

To address this challenge, Farrukh et al proposed an improvised implant formulation known as "coated" implants to enhance the bioavailability of WA.[193] The method involves coating polycaprolactone implants with 20–30 layers of polycaprolactone solution containing 0.5–2% of WA dissolved in dichloromethane. When compared with the ineffective intraperitoneal administration of the same total dose of WA, the drug-eluting implant significantly inhibited the growth of human lung cancer A549 xenografts in athymic nude mice. Another study by Ramesh et al also explored the use of polycaprolactone implants embedded with WA for controlled systemic delivery, resulting in nearly 60% inhibition of lung cancer A549 cell xenografts in mice.[141]

Low Production

Although WA can be isolated from the leaves, berry (winter cherry) and root of WS, its content is relatively low. The data showed that the concentration of major active compounds known as withanolides, represented by WA, in the leaves typically ranges from 0.001% to 0.5% of the dry weight.[194] Considering the broad pharmacological effects of WA and WS, the demand for the plant in the market continues to increase. According to data, approximately 9127 tons of dried plant material are required for the production of WA in India, while the annual yield is around 5905 tons.[195] Clearly, the current cultivation of the plant cannot meet the market demand. Therefore, increasing the production of withanolides in the plant has become a significant focus of attention.

Currently, there are various methods to increase withanolides production in plants. Firstly, concerning plant cultivation, it has been reported that compared to Kunapa jala, farmyard manure, and inorganic fertilizer, the application of Pancha gavya can increase withanolides production in plants.[196] There also are some new technologies to be developed to increase the production of withanolides in plants by optimizing conditions to enhance the accumulation of effective metabolites or secondary metabolites. Studies have shown that inducers such as 100 ppm salicylic acid, 50 ppm jasmonic acid, and 100 ppm chitosan can be sprayed on the leaf surface,[197] short-term UV-B radiation (less than 3 hours),[198] or low concentrations of WcAgNPs to improve root organogenesis,[199] all of which promote the synthesis of withanolides compounds in plants. Endophytes can also be used to regulate the expression of withanolides biosynthetic genes in plant leaves and roots,[200] overexpress squalene synthase (SQS)[201] or cycloartenol synthase,[202] or use treatments involving ultrasound, vacuum infiltration, and thiol compounds (l-cys at 100 mg/l, STS at 125 mg/l, DTT at 75 mg/l) to promote the integration and expression of the gusA gene in transgenic plants,[203] thereby increasing withanolides content in the transgenic plants. In addition, researchers have established a multiple shoot culture system of WS using single shoot apices as explants,[204] and examined the withanolides production from adventitious root cultures,[205] hairy roots,[206] and cell suspension of WS,[207,208] all of which aim to provide theoretical basis for efficient withanolides production in the industry.

Overall, the use of polycaprolactone implants embedded with WA shows promise as a potential therapeutic approach for the treatment of cancers. However, further research is needed to determine its safety, effectiveness, and production in humans before it can be used as a clinical treatment.

Conclusions and Outlook

The exploration of various plant extracts for their potential anti-tumor properties has been extensive. Among these extracts, WA, the primary bioactive component of the Ayurvedic herb WS, is a promising anti-tumor agent. While some studies have identified potential target proteins of WA, such as vimentin and heat shock proteins, the exact mechanism of action of WA and its comprehensive effects on cancer cells remain active areas of research. Understanding the systematic effects of WA on cancer cells is crucial for its development as an anti-tumor agent. A critical aspect in the development of WA as an anti-tumor agent is assessing its toxicity and safety. While animal studies have shown that WA is well-tolerated, further research is required to thoroughly evaluate its toxicity and potential side effects in humans. Safety is

paramount in the development of any therapeutic agent. Moreover, to enhance its therapeutic effectiveness, innovative approaches to improve the bioavailability of WA are needed. Methods like drug-eluting implants and other delivery systems show promise in enhancing the delivery and targeting of WA to cancer cells.

In conclusion, WA holds immense potential as an anti-tumor agent, and its pharmacological properties have shown significant antitumor efficacy in various cancers. As research in this field continues to progress, we expect a better understanding of the precise mechanisms of WA's action, its toxicity profile, and advancements in delivery strategies. These efforts will contribute to establishing WA as a potent and safe candidate for cancer treatment, opening new possibilities for clinical applications and improving the overall outlook in the fight against cancer.

Abbreviations

ACC, Adrenocortical carcinoma; AKT, Protein kinase B; Cdc25C, Cell division cycle 25C; Cdk1, Cyclin-dependent kinase 1; CRC, Colorectal cancer; CSCs, Cancer stem cells; DLBCL, Diffuse large B-cell lymphoma; DMBA, Dimethyl butanoic acid; EMT, Epithelial mesenchymal transition; ER, Endoplasmic reticulum; EGFR, Epidermal growth factor receptor;GBM, Glioblastoma; GPX4, Glutathione peroxidase 4; HCC, Hepatocellular carcinoma; HMOX1, Heme oxygenase-1; IC_{50}, Half maximal inhibitory concentration; IKK, IκB kinase; IL-6, Interleukin-6; i.p., Intraperitoneal injection; JAK2, Janus-activated kinase 2; LXR-α, Liver X receptor-α; MAPK, Mitogen-activated protein kinase; MGMT, O6-methylguanine-DNA methyltransferase; NF-κB, Nuclear factor kappa B; NSCLC, Non-small cell lung cancer; rhGas6, Recombinant human growth inhibition specific protein 6; ROS, Reactive oxygen species; SO, Sorafenib; T-ALL, Lymphoblastic leukemia; TTFields, Tumor treating fields; uPA, Urokinase-like plasminogen activator; VEGF, Vascular endothelial growth factor; WA, Withaferin A; WS, *Withania somnifera* (L.) Dunal.

Data Sharing Statement

Data will be made available from the corresponding author on request.

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