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The Potential Pharmacological Effects of Natural Product *Withaferin A* in Cancer: Opportunities and Challenges for Clinical Translation

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ABSTRACT

Cancer is one of the biggest health concerns with a complex pathophysiology. Currently, available chemotherapeutic drugs are showing deleterious side effects, and tumors often show resistance to treatment. Hence, extensive research is required to develop new treatment strategies to fight against cancer. Natural resources from plants are at the forefront of hunting novel drugs to treat various types of cancers. Withaferin A (WA) is a naturally occurring withanolide, a biologically active component obtained from the plant Ashwagandha. Various *in vitro* and *in vivo* oncological studies have reported that Withaferin A (WA) has shown protection from cancer. WA shows its activity by inhibiting the growth and proliferation of malignant cells, apoptosis, and inhibiting angiogenesis, metastasis, and cancer stem cells (CSCs). In addition, WA also showed chemo- and radio-sensitizing properties. Besides the beneficiary pharmacological activities of WA, a few aspects like pharmacokinetic properties, safety, and toxicity studies are still lacking, hindering this potent natural product from entering clinical development. In this review, we have summarized the various pharmacological mechanisms shown by WA in *in vitro* and *in vivo* cancer studies and the challenges that must be overcome for this potential natural product's clinical translation to be effective.

Introduction

Cancer has been the leading cause of death in most developed countries, next to heart disease during the past decade [1]. However, a recent analysis showed that cancer-related deaths increased by 6% and mortality of cardiovascular diseases was reduced by 4%, indicating there is a rise in the occurrence of cancer incidents [2]. According to Cancer Statistics 2019, the number of estimated cancer cases and deaths are reported as 1,762,450 and 606,880, respectively, in the United States (US) [3]. According to the WHO 2020 report, cancer accounts for 10 million deaths [4]. Abnormal cell proliferation is the main characteristic feature of cancer cells, and its pathophysiology remains complex. Chemotherapeutic drugs in clinical use also act on non-cancerous healthy cells apart from targeting cancerous cells. Apart from this, the available chemotherapeutic drugs are showing severe side effects and also facing drug-resistance issues [5]. Hence, there is a great need to discover new therapeutics to combat the problems of the current chemotherapeutic drugs. Natural resources are being used to find new medications, mostly from plant sources. Moving on to natural compounds, different phytochemicals are effective in treating cancer with more efficacy and safety [6, 7]. So far, there are a few phytochemicals that are in clinical use like vinca alkaloids (vinblastine and vincristine [8], etoposide, and taxanes (paclitaxel), which are showing very effective results in cancer treatment and are considered as chemotherapeutic drugs [9].

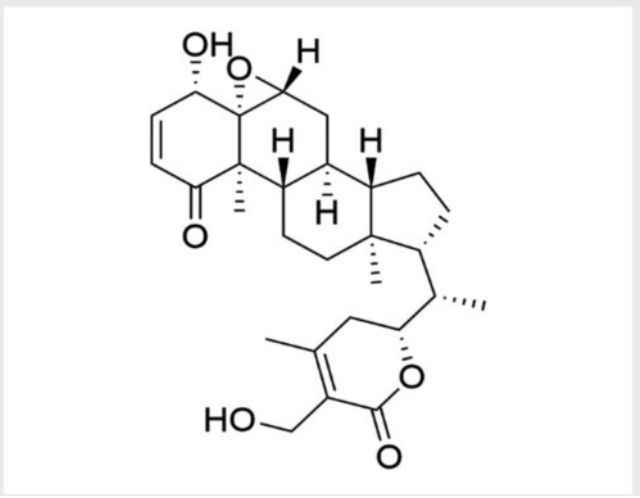
Withania somnifera (WS), commonly known as Ashwagandha or Indian ginseng, belongs to the family Solanaceae and has been reported for its various pharmacological actions since antiquity. The extractions of WS from various plant parts have been linked

ABBREVIATIONS

ALDH	Enzyme aldehyde dehydrogenase
BRSM1	Breast cancer metastasis suppressor-1
Cdc25C	Cell division cycle 25C
Cdk1	Cyclin-dependent kinase 1
CSCs	Cancer stem cells
DMBA	7,12-dimethylbenz anthracene
DR5	Death receptor 5
EMT	Epithelial-mesenchymal transition
FOXO3a	Forkhead box O3a
HDAC	Histone deacetylase
Hsp90	Heat shock protein 90
ILs	Interleukins
MAPK	Mitogen-activated protein kinase
MMTV-*neu*	Mouse-mammary tumor virus-*neu*
MSCs	Myeloid suppressor cells
MST	Mean survival time
NF-κB	Nuclear factor kappa B
PARP	Poly (ADP-ribose) polymerase
Pin1	Protein interacting with never in mitosis A1
PK	Pharmacokinetic
QSAR	Quantitative structure-activity relationship
ROS	Reactive oxygen species
SAC	Spindle assembly checkpoint
STAT3	Signal transducer and activator of transcription 3
TGF-β1	Transforming growth factor β1
TNF-α	Tumor necrosis factor α
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
VEGF	Vascular endothelial growth factor
WA	Withaferin A
WS	*Withania somnifera*
WSE	*Withania somnifera* extracts

to different biological properties such as immunomodulatory [10], anti-inflammatory [11], anti-bacterial [12], and cytotoxic activity toward cancer cells [13]. The extracts of WS were used as an energy enhancer and to build muscle mass. It is also used for enhancing memory and learning abilities [14]. This plant contains several active constituents; among them, Withaferin A (WA) has been demonstrated to possess excellent pharmacological effects, mainly immunomodulatory effects and inhibiting cancer-cell proliferation.

Methodology: The present review comes up with information on WA in various cancer studies. An in-depth literature search was conducted on WA and its related pharmacological activities in both *in vitro* and *in vivo* studies of cancer. Google Scholar, PubMed, and ScienceDirect are the search engines used to collect the literature. We have also collected data from websites, the World Health Organization (WHO) [4], and the National Cancer Institute (NCI) [9]. Withaferin A, *Withania somnifera,* cancer, anti-tumor, apoptosis, angiogenesis, pharmacokinetics, and safety were the keywords used to collect the information.



▶ **Fig. 1** Structure of Withaferin A

Structure of Withaferin A

Withanolides are the class of chemicals isolated from the leaves and roots of WS. Among all the withanolides, Withaferin A (WA) is by far the most studied bioactive compound in various *in vitro* and *in vivo* cancer studies [15, 16]. Withanolides are a group of naturally occurring steroidal compounds. The structure of WA has a C28 steroidal nucleus consisting of four cycloalkane rings. Out of the four cycloalkane rings, three are cyclohexane and one is cyclopentane, which is fused. Proper oxidation of C22 and C26 atoms resulted in the formation of a six-membered lactone ring with five carbon atoms and one oxygen atom [17]. The cytotoxic potential of WA is mainly due to the double bond at the C2–3 position, and the dissociation of this double bond resulted in decreased cytotoxic activity [18]. ▶ **Fig. 1** shows the structure of Withaferin A.

Several reports showed WA as being potent for preventing and treating cancer [19–21]. However, it remains at the budding stage for clinical use due to a lack of progress in pharmacokinetics, safety, toxicology, and clinical studies. This review summarized various pharmacological mechanisms of WA and potential pitfalls to becoming one of the effective drugs in the treatment regimen of cancer and its prevention. The IC_{50} values of WA in various studies are listed in ▶ **Table 1**.

Pharmacological Effects of Withaferin A in Cancer

Various pharmacological properties and mechanisms involved in the demonstration of the anti-tumor effects of WA are summarized in the following sections. ▶ **Fig. 2** shows a simplified diagram of the pharmacological mechanisms of WA in cancer at the molecular level.

▶ **Table 1** Summary of IC_{50} values of Withaferin A in various types of cancers and mechanisms of action in *in vitro* studies.

S. No.	Type of cancer and cell line used	IC_{50} (μM)	Mechanism shown by WA	Ref
1.	**Prostate cancer**		Apoptosis induced cell death by increasing the expression of Par-4 and activation of caspases	[33]
	PC-3 PzHPV-7 LNCaP CWR22RV-1 cells	4.00		
	PC-3	11.48	Cytotoxicity-induced cell death	[107]
2.	**Pancreatic cancer**		Binds to HSp90 and inhibits Hsp90 through an ATP-dependent manner	[20]
	Panc-1	1.24		
	MiaPaCa2	2.93		
	BxPc3	2.78		
3.	**Non-small-cell lung cancer adenocarcinoma**		Induced cell apoptosis and autophagy through ROS-dependent pathway Downregulation of mTOR/STAT3 signaling pathway	[90]
	A549	0.99		
	CL141	0.46		
	H441	0.57		
	CL97	1.49		
	H1975	0.35		
	Squamous cell carcinoma CL152	0.50		
	Large-cell carcinoma H1299	0.60		
4.	**Colon cancer**		Cytotoxic-induced cell death	[107]
	CaCo-2	11.06		
	Hepatic cancer WRl-68	8.51		
5.	**ALT**		Telomere dysfunction and upregulation of DNA damage and downregulation of c-Myc and n-Myc lead to apoptosis in cancer cells	[108]
	JF-CF 1 L	1.91		
	JF-CF 4D	1.27		
	TEP			
	JF-CF 6B	2.55		
	JF-CF 6 G	2.97		
6.	**Breast cancer**		Cell cycle arrest at the G_2/M phase induces expression of Hsp70 Degradation of Raf-1, an Hsp90 client protein	[90]
	MDA-MB-231, MDA-MB-468	1.58		
	MCF-7	0.85	Cell cycle arrest at G_2/M phase	[63]
	MDA-MB-231	1.06		
	MCF-7	8.08	Cytotoxic-induced cell death	[107]
7.	**Cervical cancer**		Inhibition of cancer cell growth by down-regulating the expression of oncogenes HPV E6 and E7 and restoring p53 tumor suppressor gene activity	[39]
	CaSki	0.45		
	Hela	1.20		
	C33a	0.20		

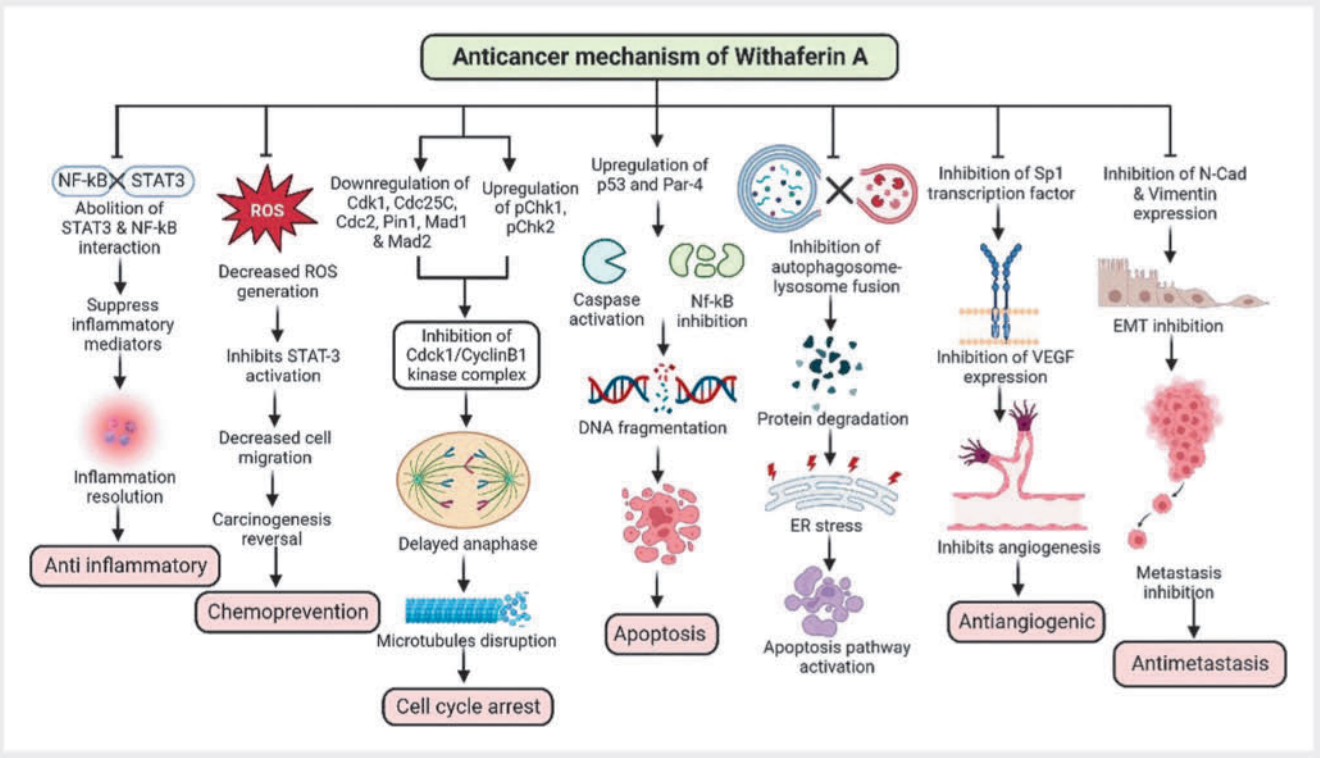
continued next page

▶ **Table 1** *Continued.*

S. No.	Type of cancer and cell line used	IC_{50} (μM)	Mechanism shown by WA	Ref
8.	HepG-2	0.21	Cell cycle arrest at the G_2/M phase and reduction in self-renewal capacity in cancer cells	[70]
	MD-MBA	0.99		
	AsPC1	0.75		
9	**Multiple melanoma**		Cell cycle arrest at the G_2/M phase and inhibition of CSC migration	[76]
	MM-CSCs (CSCs derived from multiple melanoma patients)	0.64		
10	**Thyroid cancer**		In combination with sorafenib, WA enhanced cell cycle arrest and apoptotic cell death mediated through caspase-3 activation and PARP-cleavage	[84]
	BCPAP	0.15		
	SW1736	2.50		
11	**Ovarian cancer**		Cisplatin (20 μM) + WA (1.5 μM) showed synergistic action by causing ROS generation and DAN damage	[87]
	A2780	6.00		
	A2780/CP70	4.50		
	CAOV3	5.00		
12	**Colorectal adenocarcinoma**		Cell cycle arrest mediated by degradation of Mad2, a mitotic checkpoint protein	[27]
	SW480	0.37		
	HCT116	1.27		
13	**Osteosarcoma**		Apoptotic-induced cell death through ROS-mediated reduction in mitochondrial membrane potential	[109]
	U2OS	0.32		
14.	**Myeloid leukemia**	2.00	Apoptotic-induced cell death through ROS generation and mitochondrial dysfunction	[110]
	HL-60	1.00		
15.	**Hepatocellular carcinoma**		Apoptotic-induced cell death through PARP cleavage	[45]
	HepG2 Huh7 MHCC97L	5.00		
16.	**Fibrosarcoma**		Degradation of vimentin and caspase-mediated apoptosis	[111]
	HT1080	0.40		
	Leiomyosarcoma			
	SKLMS1	0.41		
	Liposarcoma			
	PLS-1	0.53		

Induction of cell cycle arrest

WA showed a stathmokinectic (arresting of cell division at metaphase) effect on ascites tumor cells and mouse sarcoma 180 (S-180) model. WA arrested the cell division at metaphase by disrupting the microtubules of mitotic spindles, and a double membrane was found around the dispersed chromosomes. Treatment with WA at a dose of 30 mg/kg of mouse-bearing ascites tumors showed growth inhibition and the clustering of macrophages around it. WA arrested the cell cycle at the G_2/M phase and is correlated with decreasing the levels of cyclin-dependent kinase 1 (Cdk1) and cell division cycle 25C (Cdc25C) protein levels, leading to inhibition of the Cdck1/CyclinB1 kinase complex [22]. Similar results of WA-induced cell cycle arrest were reported in studies on B-cell lymphoma. Cdc2 is required for the progression of the G_2/M phase, which is reduced by WA treatment, thus arresting cells at the G_2/M phase [23]. The activation of Cdc kinase is dependent on the series of phosphorylation and dephosphorylation mechanisms. Wee1 is a protein of the kinase family that negatively regulates the function of Cyclin-B1 and Cdc. Upregulation of Wee1 ceases the Cdc2 kinase activity and cyclin-B1, consequently hindering the cells from entering into the M phase [24]. WA treatment arrested the cell cycle at the G_2/M phase in prostate cancer cells by upregulating the Wee1 protein and inhibiting Cdc2 activity [25]. In addition to Cyclin-B and Cdc protein kinases,



▶ **Fig. 2** A simplified diagram of the pharmacological effects of WA in cancer at the molecular level. WA induces cell cycle arrest at the G_2/M phase by inhibiting the cyclinB1/CDck1 complex upon activation of p21 and Wee1 protein. Caspase mediated apoptosis by WA through PARP cleavage. WA generates ROS results in the activation of JNK and leads to mitochondrial dysfunction. The cells affected by ROS undergo apoptosis. WA inhibits angiogenesis by restricting the binding of the Sp-1 gene to its promoter region of the VEGF gene. Metastasis and tumor invasion are hindered by WA through suppression of EMT and vimentin expression and inhibition of metalloproteinases.

the cell cycle is also controlled by DNA damage sensors like p-Chk1 and p-Chk2, which act as G_2/M phase checkpoints to obstruct the mutated cells from entering the M phase. In osteosarcoma cells, WA arrested the cell cycle at the G_2/M phase, which is indicated by observing increased mRNA expression of p-Chk1 and p-Chk-2 [26].

Another mechanism by which WA ceases the cell cycle is by inhibiting the spindle assembly checkpoint (SAC). SAC is a mitotic checkpoint complex consisting of Mad1 and Mad2 components, which are essential for the initiation of anaphase. Proteasomal degradation of Mad2 by WA delayed the onset of anaphase in cell lines of colorectal adenocarcinoma [27].

Protein interacting with never-in mitosis A1(Pin1) is a member of the protein family peptidyl-prolyl-isomerases and a subfamily member of parvulins. Pin1 is considered a mitotic protein that performs the major regulatory mechanism in the cell cycle. During post-translational modifications, Pin1 catalytically induces conformational changes after protein phosphorylation [28]. Increased levels of Pin1 are related to cancer cell progression in a cyclin-dependent manner [29]. Treatment with WA arrested the cell cycle at the G_2/M phase by significantly downregulating the expression of Pin1. The cell cycle arrest is mediated by reducing the protein levels of cyclin B1 [30].

Induction of apoptosis

Apoptosis is the process of programmed cell death and acts as a homeostatic mechanism to maintain cell population in tissues. The word apoptosis originated from the Greek term that means dropping off or falling off [31, 32]. Disruption of apoptosis can promote tumor initiation, progression, metastasis, and treatment resistance. In androgen-refractory prostate cancer cells, WA treatment induced apoptosis by upregulating the expression of Par-4 followed by activation of caspases and inhibition of NF-κB activity [33]. The Bcl-2 family of proteins consists of both pro-apoptotic (Bax and Bak) and anti-apoptotic (Bcl-2 and Bcl-x_L) molecules, which play a role in apoptosis through activation of the caspase pathway and mitochondrial dysfunction [34]. WA treatment showed apoptosis in breast cancer cells, which is mediated through FOXO3a and the Bim-dependent pathway and increased cytoplasmic histone-associated DNA fragmentation. WA also showed a marked increase in levels of the Bcl-2 family of proteins such as Bak, Bax, and both short and long isoforms of Bim [35].

In pancreatic cancer cells, WA showed a dose-dependent apoptosis. A 5 μM concentration of WA showed 50% cell death in pancreatic cancer cells [20]. Reactive oxygen species (ROS) have a major role in cancer, and elevated levels of ROS were reported in almost all cancer cells. ROS activate several cell signaling pathways such as MAPK (ErK1/2, p38, and JNK), Src/PKD1-dependent NF-κB activation, and PI3K/Akt signaling cascades [36]. Treatment

of human colorectal cancer cells with WA induced ROS-dependent apoptosis and upregulated p-JNK expression [37].

WA increased the G_2/M-phase population in breast cancer cells and induced apoptosis through Hsp90 inhibition. At a 5 µM concentration, WA abolished Akt phosphorylation and depletion of Raf-1, a client protein of Hsp90. The same study also demonstrated that WA strongly inhibited NF-kB p65 phosphorylation. WA showed apoptotic activity in oral Ca9-22 cell lines by activation of caspases and PARP and also caused DNA damage through ROS generation and induced γH2AX expression [38]. WA downregulated the expression of HPV E6/E7 oncogene and restored the function of p53 tumor suppressor gene function, resulting in apoptotic cell death in cervical cancer cells [39].

Autophagy induction

Autophagy is a self-conservative catabolic process, which performs the elimination of degraded cellular organelles and cytoplasm under stressful conditions like nutrient deprivation. Autophagy acts as a homeostatic mechanism to recycle cellular energy, but it has been reported in the pathophysiology of cancer by prolonging cell survival and tumorigenesis [40]. Cancer cells, being highly proliferative, require a sustained energy supply obtained from high levels of protein synthesis and degradation of cellular organs. Therefore, inhibition of autophagic degradation would be an effective target in cancer therapy. In response to cancer chemotherapy or radiation therapy, autophagy is characterized into four forms: cytotoxic, cytostatic, cytoprotective, and non-protective [41]. Most of the cancer cells sidestep the chemotherapy by undergoing cytoprotective autophagy to provide a continuous fuel supply.

An incomplete autophagic induction by WA treatment was observed in pancreatic cancer cells through inhibition of the fusion of the autophagosome with a lysosome. The accumulated degraded proteins induced endoplasmic stress, resulting in the activation of apoptotic pathways in both *in vitro* and xenograft animal models (X. Li et al., 2016). LC3-I and LC3-II (microtubule-associated protein2 light chain 3) are autophagic-specific proteins found to be accumulated in WA-treated breast cancer cells, indicating the impairment of autophagy by hindering lysosomal degradation [42]. In addition, WA also increased one more autophagic protein, Beclin-1, which is involved in autophagosome nucleation [21].

WA in combination with cisplatin induced autophagic cell death mediated by the upregulation of LC3-II and suppression of p62 [43], whereas, in combination with doxorubicin, autophagy is induced through ROS generation in ovarian cancer cells, which is further facilitated by apoptosis [44].

In contrast to the above-mentioned results, WA induced cytoprotective autophagy by inducing a complete lysosomal degradation of cytoplasmic cargo in hepatocellular carcinoma cells, halting cell death. Concurrently, WA in the presence of autophagic inhibitors like chloroquine and bafilomycin showed apoptotic cell death through PARP cleavage, which is not observed with autophagic inhibitors alone, suggesting a synergistic inhibition of cancer cell death was observed when given in combination [45].

Chemoprevention by Withaferin A

Chemoprevention is one of the novel approaches for preventing or reversing the process of carcinogenesis by using either natural or synthetic compounds including various dietary constituents. Many chemotherapeutic drugs fail to prevent and completely cure cancer. Hence, the field of chemoprevention is emerging to compensate for the problems or challenges faced with the current chemotherapeutic drugs and to improve the patients' survival rates.

Most of the chemopreventive agents for cancer are isolated from natural sources. These agents work by either preventing the initiation of mutagenesis or preventing the further progression of cancer. WA is found to be a protective agent in several cancer-induced studies. STAT3 activation is one of the oncogenic signaling pathways that promotes tumor growth. Increased leptin levels are highly correlated with the activation of STAT3 signaling in breast cancer [46]. WA showed inhibition of leptin-induced STAT3 activation in breast cancer cells (MCF-7 and MDA-MB-231). WA also showed suppression in colony formation and cell migration of cancer cells induced by leptin [47]. WA given orally at a dose of 20 mg/kg has shown a protective effect in 7,12-dimethylbenz [3] anthracene (DMBA)-induced oral carcinogenesis in hamsters. The underlying protective mechanism against carcinogenesis is a reduction in the levels of ROS at the early stages of carcinogenesis [48].

Oral doses of 3 and 4 mg/kg of WA showed chemopreventive effects in a transgenic model of $APC^{Min/+}$ mouse and azoxymethane/dextran sodium-sulphate-induced intestinal and colon cancer. At the initial stages of carcinogenesis, WA significantly reduced the expression of ki-67, which is associated with inflammation and proliferation of tumor cells [49]. Therefore, it is expected that supplementation of WA or WA-containing extracts may produce promising cancer preventive effects in certain populations who are at risk of developing different types of cancers due to pre-existing conditions, mutation, exposure to a carcinogenic environment, etc.

The potential advantages of WA over current anticancer drugs emphasize its promising role in possible cancer management. The majority of the existing literature indicates that WA demonstrates superior efficacy compared to traditional chemotherapeutic drugs. Based on the data from preclinical studies and early phase clinical trials, we observe a higher rate of tumor response and increased patient survival rates with WA administration. One of the key strengths of WA lies in its ability to minimize the adverse effects commonly associated with conventional chemotherapy. Our comparative analysis reveals a significantly reduced incidence of severe side effects, such as nausea, hair loss, and immunosuppression, making WA a more tolerable and patient-friendly option. WA introduces a paradigm shift in cancer therapy by offering a more streamlined and patient-centric approach to treatment administration. Unlike complex and invasive chemotherapy regimens, WA allows for simpler administration protocols, potentially leading to improved patient compliance and overall treatment outcomes. Beyond the conventional metrics of comparison, WA introduces innovative features that set it apart in the realm of cancer therapy. These may include targeted drug delivery mechanisms, personalized treatment approaches, or novel modes of ac-

tion that address specific challenges posed by existing chemotherapeutic agents.

By highlighting these aspects, our comparative analysis aims to establish WA as not only a viable alternative but also a groundbreaking and important option in the evolving field of cancer therapy. We believe that understanding the distinct advantages of WA will contribute significantly to its recognition and adoption within the medical community, ultimately advancing the landscape of cancer treatment.

Radiosensitization effects of Withaferin A

Despite radiation therapy being predominant in the treatment of solid tumors, pre-exposure with a chemotherapeutic agent enhances the sensitivity of cancer cells toward radiation. Radiosensitization is a target-based approach for selective cytotoxicity to tumor cells over normal cells. The compounds that sensitize the cancer cells to radiation are known as radiosensitizers. WA enhanced the apoptosis in human lymphoma U937 cells when exposed to X-ray irradiation. Apoptotic death is driven by the suppression of Bcl-2 expression and activation of the JNK pathway [50]. The combination of WA with radiation resulted in an increased generation of ROS when compared with radiation therapy alone in Caki cells. Caki cells when exposed to radiation showed inhibition of Akt signaling in 15 min, but a recovery was observed in 90 min of post-treatment followed by an increase in Akt activation. When WA was given as a co-treatment along with radiation, sustained inhibition of Akt activity was observed, which later recovered weakly [51].

Very promising results of WA were obtained in the mouse Ehrlich ascites carcinoma model in combination with gamma radiation. WA at a dose of 30 mg/kg twice a day and 7.5 Gray (Gy) radiation exposure showed improvement in the tumor-free survival of mice [52]. A trimodality treatment consisting of radiation (40 Gy), WA (40 mg/kg), and hyperthermia (43 °C) in the xenograft model of B16F10 melanoma in C57BL mice produced a high degree of delay in tumor growth and increased mean survival time (MST). The combination of radiation and WA showed similar results, suggesting that WA is a good radiosensitizer [53].

Effect of Withaferin A on EMT and metastasis

Epithelial mesenchymal transition (EMT) is a transition process in which the epithelial cells transform into mesenchymal cells and become invasive and migrant. This EMT is a fundamental process and is essential for tissue generation and organ formation during embryogenesis. However, its role is also found in pathological conditions of fibrosis and tumor metastasis [54]. E-cadherin is an epithelial marker that is suppressed during EMT, and there is an increase in markers of mesenchymal origin such as N-cadherin and vimentin. In the MCF-10A cell line, WA partially reversed the TGF-β1- and TNF-α-induced EMT. When H1299 and A549 cell lines were pre-treated with 0.5 µM of WA, inhibition of EMT induced by TGF-β1 and TNF-α was observed, coupled with an increase in E-cadherin marker expression [55].

Vimentin is a cytoplasmic intermediate filament protein, and its expression in cancer cells is associated with EMT, aiding in local invasiveness and metastasis [56]. In detached cancer cells, vimentin is highly present and provides motility for the migration of tumor cells. In the MDA-MB-231 xenograft model, WA showed an anti-metastatic effect through inhibition of vimentin expression [57]. WA inhibited vimentin assembly by directly binding to cysteine 328 residue through covalent modification and also inhibited proteasomal activity in a vimentin-dependent manner in the case of breast cancer [58].

During carcinogenesis, malignant cells interact with the microenvironment of the tumor. Tumor cells undergoing invasion and metastasis must degrade the physical barriers around them. Metalloproteinases are zinc-dependent endopeptidases that have a role in the degradation of extracellular matrix components during cancer progression [59]. Metalloproteinases promote cancer cell proliferation by releasing growth factors such as insulin-like growth factors and ligands for epidermal growth-factor receptors [60]. Upon treatment with WA, decreased mRNA expression of metalloproteinases was observed, which resulted in inhibition of invasiveness and metastasis in human cervical cancer Caski and SK-Hep1 cells. The matrix metalloproteinase inhibition is observed to be mediated through suppression of the Akt pathway [61].

Regulatory mediators such as genes have a major contribution in coordinating metastasis. Metastasis suppressors find their way in inhibiting tumor cell migration. Breast cancer metastasis suppressor-1 (BRSM1) highly controls the transcription of genes associated with metastasis. BRSM1 controls gene transcription of fascin, epidermal growth factor receptor, and osteopontin, which are involved in several steps of metastasis [62]. A study reported the upregulation of BRSM1 expression upon treatment with WA in metastatic breast cancer cells, which also decreased the cancer cell invasiveness [63].

Anti-angiogenic effects of Withaferin A

Cancer cells undergo necrosis when they are deprived of nutrients and oxygen. Proper blood supply is required for the growth of neoplasm and metastatic progression. Hence, cancer cells stimulate the development of angiogenesis when they suffer an inadequate supply of nutrients and oxygen for their survival [64]. Vascular endothelial growth factor (VEGF) is the chief pro-angiogenic protein involved in the stimulation of angiogenesis in tumor tissue via activating chemical mediators. Docking studies showed WA has a strong binding capacity toward VEGF, and this binding is highly similar to the commercially available anticancer drug Bevacizumab [65].

When WA was applied on the chorioallantoic membrane to evaluate the anti-angiogenic effects, it inhibited neovascularization in the developing chick embryo. The same study also reported the inhibition of VEGF expression by WA at a dose of 7 mg/kg in the Ehrlich ascites mouse tumor model. The decreased expression of VEGF by WA is mediated through the inhibition of Sp1-transcription factor binding to the VEGF-gene promoter region. An *in vivo* liver cancer model in nude mice showed the down-regulation of the expression of VEGF protein by WA and damaged the vasculature around the tumor tissue. The investigation revealed that treatment with WA reduced the expression of Pyk2 protein, which is involved in the invasiveness of tumors [66]. Lowered hemoglobin levels were observed in the fibroblast growth

factor Matrigel plug isolated from the animals upon treatment with WA, indicating its anti-angiogenic property [67].

Effect of Withaferin A on cancer stem cells

Cancer stem cells (CSCs) are the main reason behind the relapse of cancer after chemotherapy because the malignant cells attain self-renewal from the few long-lived CSCs. Chemoresistance for most of the drugs is mainly due to the high expression of anti-apoptotic proteins, efflux transporters, and cell repair enzymes in CSCs [68]. CSCs also play a critical role in the migration of tumor cells in malignant types as seeding and colonization of cancer cells are required to form secondary tumors in different organs [69]. To combat the problem of relapse and to overcome chemoresistance, targeting CSCs would be the potential strategy.

WA at a 0.75 µM concentration inhibited the expression of the Oct-4 gene in a sphere-formation assay of pancreatic cancer stem cells, which suggests inhibition of the self-renewal capacity of CSCs as the Oct-4 gene is a key regulator of sustaining the pluripotency of stem cells and prevents the apoptosis. Forty-eight hours post-treatment with WA inhibited NANOG, a transcription factor involved in the chemoresistance and self-renewal capacity of CSCs [70]. It is believed that most of the carcinoma cells exhibit two types of cell population, adherent and floating cells. Floating cells consist of a dense population of CSCs and spontaneously form tumor aggregates or spheres [71]. WA inhibited the sphere formation and induced apoptosis of the F cell population in the UP-LN1 lymph node metastatic cell line (gastrointestinal carcinoma cell line). One more reason for hiding the apoptosis by WA is the lower levels of glutathione in the F cell sub-population [72].

Enzyme aldehyde dehydrogenase (ALDH) catalyzes the oxidation process of aldehydes through an $NADP^+$-driven manner in CSCs. There are 19 kinds of ALDH isoforms in existence. Among them, ALDH1 has been reported for its high expression in the CSCs of most cancers [73]. ALDH1-positive cells were markedly decreased after treatment with 2 mg/kg of WA on the surface epithelium, as well as on the cortex region of the ovarian cancer spheroid. Furthermore, WA halted the expression of securin, a pituitary transforming gene that is co-expressed with other CSC markers like Oct-4, CD24, CD44, ALDH1, and Shh in all kinds of ovarian cancers [74].

WA was found to be a potent inhibitor of CSCs in induced pluripotent stem cells by abrogation of tumorigenicity. β-galactosidase is a hydrolase enzyme and a marker for senescent cells. WA treatment showed an increase in senescence-associated β-galactosidase activity [75]. WA hindered the migration of CSCs derived from multiple melanoma patients and caused apoptotic cell death [76]. Decreased tumor burden and formation of mammospheres were observed during analysis of therapy-resistant CSCs in transgenic MMTV-*neu* (mouse-mammary tumor virus-*neu*) mice by WA at a dose of 0.1 mg thrice in a week for 28 days [77].

Targeting inflammation in tumor microenvironment

Inflammatory mediators like tumor necrosis factor (TNF-α) and interleukins (IL) have major contributions to cancer progression. The presence of immune cells in the tumor microenvironment induces the inflammatory response, and tumor tissue is highly supplemented with myeloid suppressor cells (MSCs) and regulatory T-cells [78]. WA has been reported for its anti-inflammatory action in various pathological diseases. Upon activation of inflammatory mediators such as IL-6 and TNF-α on tumor cells, STAT and NF-κB get activated and their interaction results in a transcriptional process for the proliferation, survival, and invasiveness of cancerous cells. WA, being anti-inflammatory, abolished the activation of STAT3 and its interaction with NF-κB by reducing the telomerase activity [79].

MSCs are immature myeloid cells in the bone marrow that migrate to the tumor microenvironment by the tumor-associated cytokines. Pro-inflammatory mediators act on immature myeloid cells and transform them into MSCs. Additionally, MSCs get activated by STAT3 and NF-κB signaling [80]. WA modulated the potency of MSCs by indirectly suppressing the pro-inflammatory mediators and downregulation of STAT3 and the NF-κB function [81]. WA abrogated the IL-6-induced STAT-3 transcriptional activity in HCT116 cells [82]. WA ameliorated pro-inflammatory mediators in ovarian-cancer-induced cachexia by suppressing NF-κB activation and nuclear translocation of p65. The inactivation of NF-κB is observed through the activation of the IKK complex, the inhibitor of NF-κB [83]. Another important dimension of WA is its potential vimentin inhibitory effects.

Combination studies

WA in combination with other chemotherapeutic drugs showed the most effective results in cancer therapy. Co-administration of WA showed synergistic action in most of the studies and reduced the chemotherapeutic dose to half, which eventually lowered the toxicity to normal cells. Sorafenib and WA potentiated the cell cycle arrest at the G_2/M phase and enhanced apoptosis through caspase-3 activation and PARP cleavage [84]. The combination of two naturally occurring compounds, sulforaphane and WA, regulated the epigenetic mechanisms involved in cancer. Sulforaphane at 5 µM and WA at 1 µM showed synergistic inhibition of histone deacetylase (HDAC) and decreased the transcription of DNA methyltransferases in both estrogen receptor-positive (ER (+)) and -negative (ER (−)) cancer cells. These concentrations have not shown any toxicity in normal MCF10A cell lines, indicating the safety of the combination [85]. Sulforaphane and WA arrested the cell cycle at the G_1 phase, and reactivation of the p21 tumor suppressor gene through an epigenetic mechanism in breast cancer cell lines was observed [86].

Cisplatin, a platinum-bearing compound, is well known for its effectiveness in treating breast and ovarian cancer, yet side effects such as toxicity toward normal cells and resistance are frequently faced issues. The IC_{50} of cisplatin in the ovarian cancer cell line was found to be 40 µM. When combined with WA at 1.5 µM, the IC_{50} is reduced to 12 µM, and this combination proved very effective in treating cisplatin-sensitive (A2780, CAOV3) and -resistant (A2780/CP70) ovarian cancer cells [87]. The suboptimal dose of cisplatin and WA showed decreased tumor burden in metastatic ovarian cancer and also eliminated CSCs [88]. The anti-tumor activity was doubled when oxaliplatin was given along with WA by suppressing the PI3K/Akt pathway in pancreatic cancer [89]. WA and cisplatin at a ratio of 1 : 10 inhibited the activity of CSCs by acting on many targets of the mTOR/STAT3 signaling pathway in non-small-cell lung cancer [90].

An anthracycline compound, doxorubicin, has been in use to treat a wide spectrum of cancers. However, its use is restricted due to its high myocardial toxicity. When doxorubicin was used in combination with WA, even at a 1 mg/kg dose, they showed effective ROS-mediated autophagic cell death in the xenograft model of ovarian cancer [44]. In the 3D *in vitro* spheroid model of ovarian cancer, the combination of DOXIL, a liposomal form of doxorubicin, with WA synergistically reduced the expression of ALDH protein [74].

Tumor necrosis-factor-related apoptosis-inducing ligand (TRAIL) has been studied for anticancer activity and found to be very effective at inducing apoptosis, but its resistance toward apoptosis is observed in a few types of cancers [91]. Co-treatment with WA sensitizes the cancer cells to TRAIL-induced apoptosis. The apoptosis mediated through the generation of ROS signals resulted in up-regulation of death receptor 5 (DR5) [92]. Though there are few studies, it is expected that WA may produce additive or synergistic effects in combination with different standard anticancer drugs, which may result in increased pharmacological effects with much better safety profiles. However, the future looks very bright for such kinds of combination approaches, and according to the authors' opinion, such kinds of studies may unveil the much better promising anti-tumor effects of WA.

Pharmacokinetic and safety studies

Pharmacokinetic (PK) and safety studies are essential to better achieve the therapeutic efficacy of any drug molecule and to enter clinical studies. PK studies are considered an integral part of the drug development process. When it comes to natural products, PK studies aid in the preparation of suitable formulations or suitable derivatives, which will aid in improved PK profiles. WA showed rapid oral bioavailability at a dose of 1000 mg/kg of WS extracts (WSE) administered orally to the mice. The 1000 mg/kg of WSE is equivalent to 0.4585 mg/kg of WA [93]. However, *in vitro* bioavailability studies in MDCK cells showed WA has poor permeability when compared to WA given in extract form, suggesting the complexity in the absorption of WA, which indicates the need to carry out detailed PK studies [94]. The time to reach maximum mean plasma concentration (T_{max}) was found to be 20 min [93]. WA, when given through the intraperitoneal route at a dose of 4 mg/kg to mice, the C_{max} of 1.8 µM was achieved in 4.98 min. The half-life ($t_{1/2}$) was reported as 1.3 h and $AUC_{0\text{-}t}$ as 541.3 ng h/ml. The same study reported the clearance of WA from plasma at a rate of 2.5 ml/min, and it was undetectable in the blood after 24 h of administration [58]. If we carefully observe the reported PK studies of WA or its plant extract, it is evident that the oral bioavailability of WA is still ambiguous. Furthermore, most of the *in vivo* cancer studies reported with WA were observed to follow the parenteral route of administration; the probable reason could be the uncertainty of its oral bioavailability. On the other side, for natural products to explore for clinical translation, the oral route is the most preferred route, and most of the natural products fail to reach the clinical application stage due to limited oral bioavailability profiles. Probably, WA may also fall into this category; hence, detailed PK and metabolic studies are warranted to better poise this interesting natural molecule for cancer therapy and chemoprevention.

The safety studies in advanced-stage high-grade osteosarcoma patients showed no toxicity at the maximum dose of 216 mg of WA in WSE. Liver enzyme elevation was reported as grade-1 severity in 38.46% of patients [95]. Molecular docking and QSAR studies predicted the toxicity of WA based on fragments present in the structure and reported a medium risk associated with higher doses [96]. Nevertheless, the toxicity studies may not be of prime need to explore novel or natural-product-based anticancer agents. The reason behind this relaxation in the requirement of toxicity studies is the severity of malignancy and the universal belief that small molecule-based anticancer drugs are not free from toxicity concerns. Moreover, since anticancer drugs are not used for long terms, the detailed battery of toxicity studies may not be of much importance compared to another category of drugs like antidiabetic or antihypertensive drugs.

Challenges Faced by WA to Become an Effective Drug in Cancer Therapy

Numerous studies reported the promising anticancer effects of WA in both *in vitro* and *in vivo* conditions. However, WA remains at the pre-clinical stage due to the few factors that are listed below and in ▶ **Fig. 3**

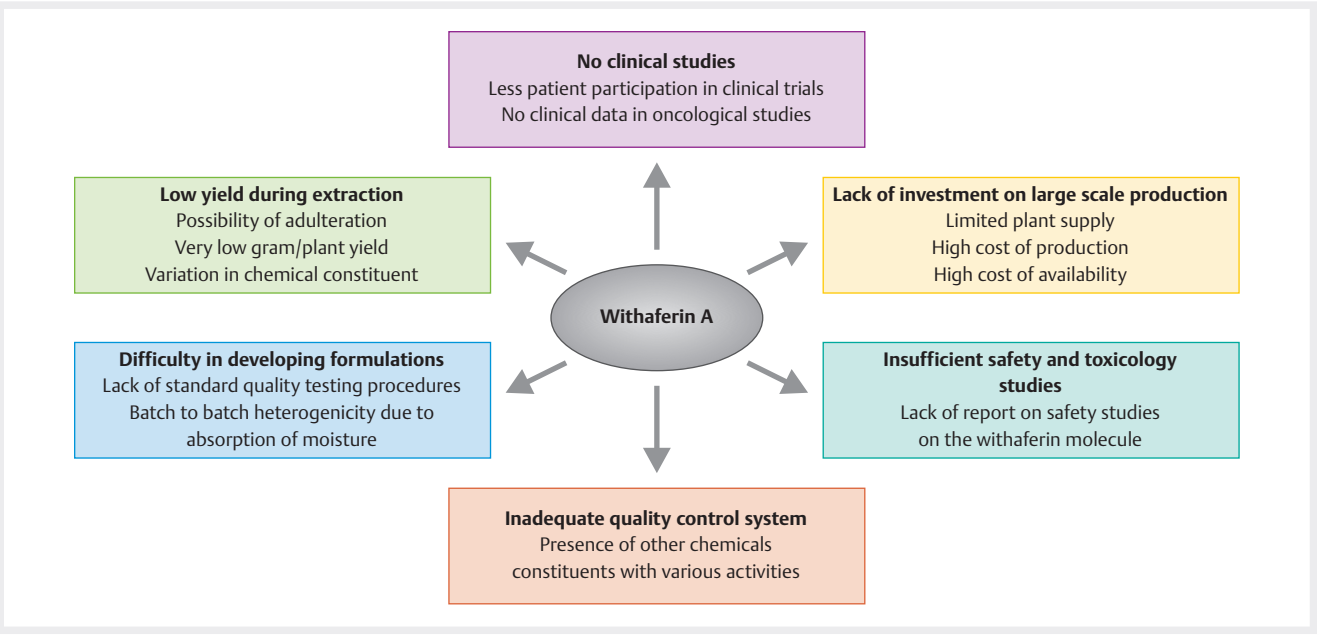
Lack of safety and toxicology studies

Every new compound should undergo a safety assessment before entering clinical studies. Safety pharmacological studies are conducted to assess whether the drug is safe or producing any toxicity at therapeutic concentrations [97]. Drugs obtained from natural sources are found to be safe; yet safety concerns are a must to save the lives of clinical trial patients. WA, being a potent anticancer molecule, remained at the developmental stage due to a lack of proper safety pharmacology studies. So far, conducted safety studies are on the extracts of WS isolated from various plant parts but not on the core molecule of WA [95]. The extracts of WS contain other withanolides that also have pharmacological activities, which creates confusion that the safety may be due to the presence of other constituents present in the extract.

Besides safety pharmacology, toxicology data have a high concern in the drug developmental stage before use on the human population. Toxicity studies provide information regarding the drug's toxic effects at and above therapeutic doses. Animal toxicity studies are very helpful in predicting toxicity in humans [98]. The current established models in cell lines, as well as in animals, are not enough to provide reliable information about the toxicity of the compound. Furthermore, anticancer drugs are well known for their cytotoxicity, and new approaches need to be discovered to predict the toxicity in humans.

Need for clinical trials

Consistently, clinical studies remain a keystone for the drug development process. Without clinical trials, no drug can enter the market, even if it is most effective. Before going to the clinical phase, pharmaceutical concepts like off-target physiological effects, pharmacokinetic and pharmacodynamic properties, therapeutic windows, shrinkage capacity of the tumor, and maximum



▶ **Fig. 3** Challenges facing WA to enter into a cancer treatment regimen.

tolerated dose are very essential to be evaluated. But unfortunately, there is no such accumulated data for WA in the case of oncology studies.

Standardization needs to be performed for natural products before entering the initial clinical phase to ensure the substance is the same in every evaluation. Most of the natural products fail the global acceptance due to a lack of a standard quality profile. The quality is being affected by adulteration and variability in the constituents in most of the extracts obtained from natural sources. Quality testing needs to be conducted at all stages, starting from collection of plant parts to the final product [99]. Clinical investigative procedures like placebo-controlled, randomized, and double-blind studies have to be performed for every new compound, which can be carried out only when the standard is available [100].

New treatment regimens and their development for curing cancer are very crucial in the present scenario to improve the patient survival rate. However, clinical trials in oncology are very complex because of the high heterogenicity of the disease. Oncology clinical studies majorly depend on several volunteers participating in the experiment. During drug development, generally healthy volunteers participate in the phase I trial, whereas most of the anticancer drugs are often likely to be more toxic and with severe side effects, and phase I and phase II trials predominantly recruit cancer patients with advanced stages and pre-treated conditions who are more likely to fail with the treatment. Most of the oncologists referring the patients to participate in the clinical studies are very low. This may be due to the lack of trust between investigators and physicians, communication between patients and physicians, low incentives for referrals, and low patient access to information [101].

Difficulties in large-scale production

Factors like authentication, geographical location, season and time of harvesting, methods of harvesting, and protocols used for the collection of plant parts are major sources for variation in the chemical constituents of the plant [102]. A high concentration of WA is found between the plant stages before the start of flowering and production of fruits, thereby gradually decreasing the concentration of withanolides [103]. Geographical location has a greater impact on the composition of pharmacologically active constituents. WS plants collected from various regions of India showed a larger variation in the root yield in terms of g/plant, which indicates that geographical location and environmental conditions influence the composition of phytochemical constituents. Among the collected plants, only a few are predominant with WA in root and others with remaining withanolides, whereas the WA content is high in the leaves, which are collected from the northern region [104]. The obtained yield of WA from the 100 g of leaves is reported as less than 2 g, which is fairly difficult for large-scale production, and this results in a supply of WA at a higher price. For example, the cost of 5 mg of WA is $44,000 from Sigma and Santa Cruz. Understanding the complexity of the variability of the plant constituents is an important measure in determining the quality, safety, and integrity of natural products [105].

One more issue with natural products is the moisture content. Raw materials obtained from plant sources have an inherent nature of moisture absorption when handled in bulk quantities, leading to heterogeneity from batch to batch [106]. The annual production of the WS plant is 1500 tons, whereas the demand is for 7000 tons, meaning there is a limited supply of plants to meet the needs of the production of raw material [102]. Because of low yield, the limited supply of plants, and the high cost of production, most pharmaceutical companies lack interest in investing

in natural products, creating obstacles in the drug discovery pipeline.

Conclusion

New drug development is the requisite for cancer treatment to overcome the problems of the current chemotherapeutic drugs in clinical use. WA is an active constituent of WS and has been reported for its anti-tumor properties in various studies. WA showed tumor regression in many cancers like colorectal, breast, thyroid, pancreatic, prostate, lung, hepatic, ovarian, and cervical cancer. WA inhibits tumor growth by inducing cell cycle arrest at the G_2/M phase, which is accompanied by upregulation of BCl-2 proteins for apoptotic cell death. WA also inhibits angiogenesis by targeting VEGFR and tumor migration through ceasing EMT. In addition to these, WA has the potency to sensitize the cancer cells during chemo and radiation therapies. Being a potent cancer-inhibitory molecule, WA is still in the development phase for various reasons. Pharmacokinetic, safety, and toxicological studies need to be conducted. New technologies and methods ease the development of WA formulations.

Contributors' Statement

Geetanjali Devabattula collected the literature and drafted the manuscript. Biswajit Panda and Rachana Yadav contributed for designing images and collection of literature. Critical revision of manuscript by Chandraiah Godugu.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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