A Comprehensive Review and Perspective on Anticancer Mechanisms of Withaferin A in Breast Cancer

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ABSTRACT

Withaferin A (hereafter abbreviated as WA) is a promising anticancer steroidal lactone abundant in a medicinal plant (*Withania somnifera*) native to Asia. The root/leaf extract of *Withania somnifera*, which belongs to the *Solanaceae* family, continues to be included in the Ayurvedic medicine formulations of alternative medicine practice. Numerous chemicals are detectable in the root/leaf extract of *Withania somnifera* [e.g., withanolides (WA, withanone, withanolide A, etc.), alkaloids, sitoindosides, etc.], but the anticancer effect of this medicinal plant is largely attributed to WA. Anticancer effect of WA was initially reported in the early 70s in the Ehrlich ascites tumor cell model *in vitro*. Since then, numerous preclinical studies have been performed using cellular and animal models of different cancers including breast cancer to determine cancer therapeutic and chemopreventive effects of WA. Chemoprevention, a word first introduced by Dr. Michael B. Sporn, was intended to impede, arrest, or reverse carcinogenesis at its earliest stages with pharmacologic agents. This review succinctly summarizes the published findings on anticancer pharmacology of WA in breast cancer focusing on pharmacokinetic behavior, *in vivo* efficacy data in preclinical models in a therapeutic and chemoprevention settings, and its known effects on cancer-relevant cellular processes (e.g., growth arrest, apoptosis induction, autophagy, metabolic adaptation, immune function, etc.) and molecular targets (e.g., suppression of oncogenes such as estrogen receptor-α, STAT3, etc.). Potential gaps in knowledge as well as future research directions essential for clinical development of WA for chemoprevention and/or treatment of breast cancer are also discussed.

Introduction

Breast cancer is a serious health problem affecting hundreds of thousands of women worldwide. In the United States alone, more than 40,000 women are expected to lose battle to breast cancer in the 2020 (1). Novel therapeutic and preventative strategies are still needed to decrease the mortality and suffering from this disease. Extracts of medicinal plants or their small molecule constituents continue to be investigated as novel strategies for therapy and/or chemoprevention of breast cancer. *Withania somnifera* (also known as Indian winter cherry or Ashwagandha) belonging to the *Solanaceae* family of plants is an appealing medicinal plant under intense investigation for its effect on cancer and other ailments. The root/leaf extract of *Withania somnifera* continues to be included in formulations of Ayurveda, Siddha, and Unani medicine practices in India and surrounding countries (2–7). More than 15 clinical trials using *Withania somnifera* extract are listed on the ClinicalTrials.gov for different conditions. Clinical effects of *Withania somnifera* extract have been studied for management of male reproductive functions, neuroprotective potential, relief from stress and anxiety, improvement of memory and cognitive functions, muscle strength and recovery, etc. (8–11). *Withania somnifera* extract is available over the counter in the United States as a dietary supplement.

The phytochemical composition of *Withania somnifera* extract is quite diverse as illustrated by existence of withanolides, alkaloids, and sitoindosides (2). The anticancer potency of every identified chemical component of *Withania somnifera* extract is yet to be determined, but withaferin A (WA; structure is shown in **Fig. 1A**), a member of the withanolide family, has been studied most extensively for anticancer effect in different cancers including breast cancer (12–18). This review summarizes the robust literature on anticancer effects of WA and its pharmacology focusing on pharmacokinetic behavior, *in vivo* efficacy data in preclinical rodent models of breast cancer, and its known effects on cancer-relevant cellular processes (e.g., cell-cycle arrest, induction of apoptosis and autophagy, metabolic adaptation, and immune function) and molecular targets (e.g., suppression of estrogen receptor-α (ERα), STAT3, etc.). Gaps in knowledge as well as future research directions to facilitate clinical development of WA for chemoprevention and/or treatment of breast cancer are also discussed.

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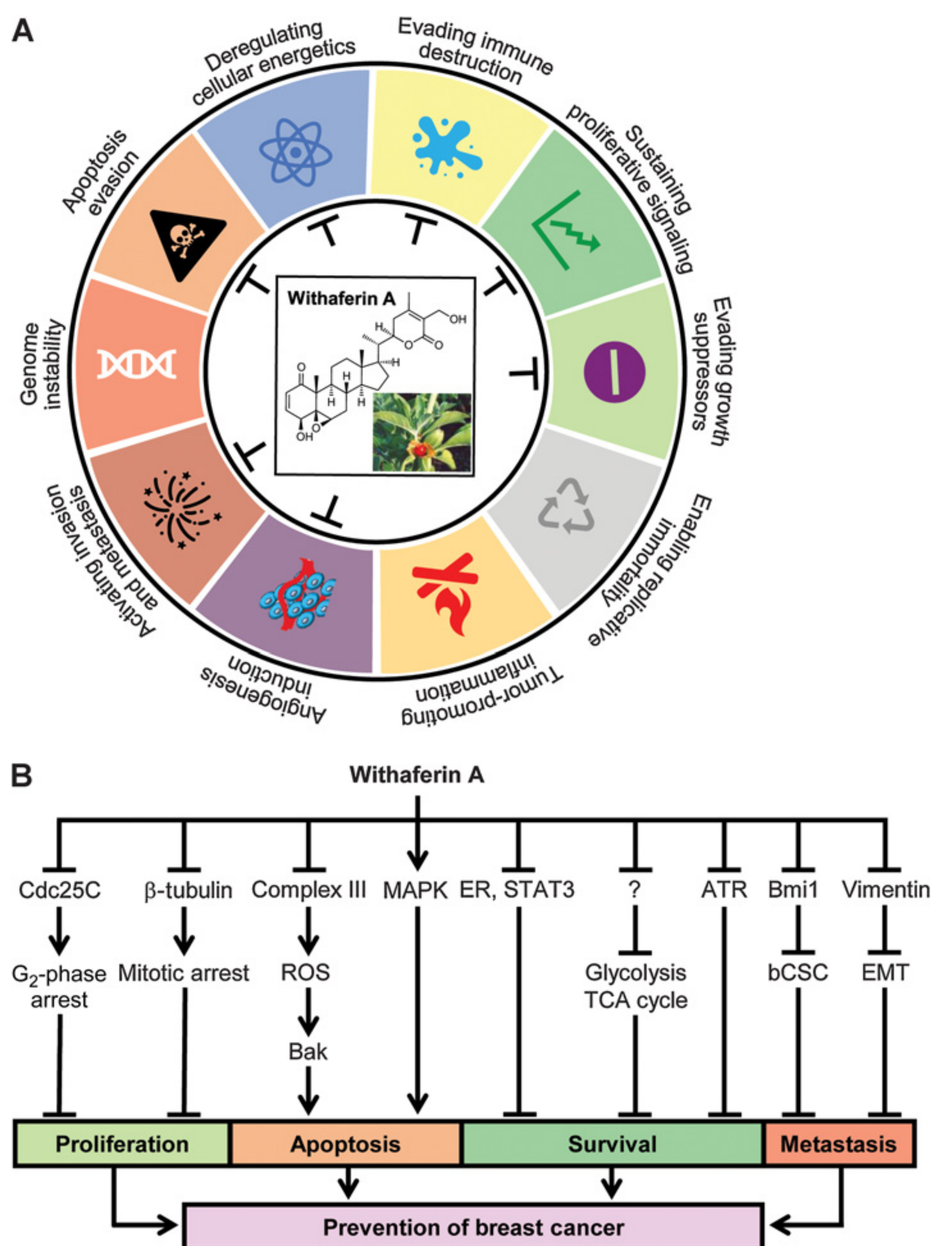

Figure 1.
A, Cancer hallmarks affected by WA treatment in breast cancer cells *in vitro* and/or *in vivo*. This figure is modified on the basis of revised cancer hallmarks reported by Hanahan and Weinberg (45). **B,** Mechanism underlying anticancer effect of WA in breast cancer cells. TCA cycle, tricarboxylic acid cycle.

Pharmacokinetic Behavior of WA

Knowledge of the pharmacokinetic behavior of an agent is of paramount importance for its clinical development. This information is critical not only for *in vitro* mechanistic studies (e.g., dose selection based on maximum plasma/serum achievable level to avoid use of supra-pharmacologic concentrations) but also for dosing schedule in preclinical and clinical studies based on half-life and tissue availability and clearance. Data on pharmacokinetic behavior of WA in rodents are summarized in **Table 1**. In female Balb/c mice, a single intraperitoneal injection of WA at a dose of 4 mg/kg body weight resulted in maximum plasma concentration (C_{max}) of about 1.8 μmol/L with half-life of about 1.3 hours (18). An exposure AUC_{0-t} of 1.09 μmol/L×hour was estimated from this study (18). These results were obtained using reverse-phase LC/MS-MS (18). In another study using LC/MS-MS, pharmacokinetic parameters for WA were determined after oral administration of aqueous extract of *Withania somnifera* root to female Swiss albino mice at a dose of 1,000 mg/kg (19). The C_{max} of WA was found to be lower [16.7 ± 4 ng/mL with observed T_{max} (time to reach C_{max}) of 20 minutes; ref. 19] than that observed after intraperitoneal injection (18). Lower plasma level of WA from oral administration of the aqueous *Withania somnifera* extract is understandable because WA is a hydrophobic molecule and hence water is not the best solvent for its extraction. Our laboratory was the first to demonstrate mammary tumor tissue

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Table 1. Pharmacokinetics of WA in mice and rats.

Dose/route	Specimen	$t_{1/2}$ (hour)	T_{max} (hour)	C_{max} (μmol/L)	$AUC_{(0-t)}$ (μmol/L×hour)	Ref.
4 mg/kg WA/i.p.	Mouse plasma	1.3	0.083	1.8	1.09	(18)
1 g/kg WSE/i.g.[a]	Mouse plasma	0.999	0.333	0.0355	0.056	(19)
4.5 mg/kg WA/i.v.	Rat plasma	0.93	0.33	0.062	0.060	(22)
4.5 mg/kg WA/i.g.	Rat plasma	1.15	0.86	0.046	0.052	(22)
1.5 mg/kg WA/i.g.	Rat plasma	0.78	1.03	0.031	0.035	(22)
0.5 mg/kg WA/i.g.	Rat plasma	1.00	0.97	0.013	0.025	(22)
5 mg/kg WA/i.v.	Rat plasma	4.5	—	6.477	7.682	(21)
10 mg/kg WA/i.g.	Rat plasma	7.6	0.11	1.315	4.983	(21)
4.5 mg/kg WA/i.g.	Rat stomach	1.02	1.50	5.679	0.030	(22)
4.5 mg/kg WA/i.g.	Rat intestine	3.21	1.80	2.826	0.025	(22)
4.5 mg/kg WA/i.g.	Rat heart	1.55	3.50	3.842	0.029	(22)
4.5 mg/kg WA/i.g.	Rat liver	0.95	3.00	1.990	0.018	(22)
4.5 mg/kg WA/i.g.	Rat lung	1.02	3.50	3.835	0.029	(22)
4.5 mg/kg WA/i.g.	Rat kidney	0.79	5.00	3.633	0.027	(22)
4.5 mg/kg WA/i.g.	Rat spleen	0.61	3.50	2.083	0.022	(22)

Abbreviations: i.g., oral; i.p., intraperitoneal; i.v., intravenous.
[a]WSE, aqueous extract of *Withania somnifera* (dosing equivalent to about 0.46 mg WA/kg body weight).

bioavailability of WA after cumulative intraperitoneal injections of 4 or 8 mg/kg body weight (five times/week for 10 weeks) by using LC/MS-MS (20). The mammary tumors in this study were induced by a single intraperitoneal injection of the carcinogen *N*-methyl-*N*-nitrosourea (MNU) to female Sprague-Dawley rats (20). The WA was also detectable in the rat plasma, but this study was not designed to determine the pharmacokinetic parameters (20). More recently, two different groups of investigators have determined pharmacokinetics and oral bioavailability of WA in rats with widely differing conclusions (21, 22). Both these studies utilized LC/MS-MS technique to measure WA levels (21, 22). Dai and colleagues (21) reported an oral bioavailability of about 32% based on WA measurement in the plasma of male Sprague-Dawley rats following oral and intravenous administrations of 10 and 5 mg/kg, respectively (21). The C_{max} for WA following intravenous and oral administrations were about 6.5 and 1.3 μmol/L, respectively (21). In the other study, pharmacokinetic parameters of WA were determined in Sprague-Dawley rats (sex not specified) after a single intravenous administration of 4.5 mg/kg or a single oral treatment with 0.5, 1.5, and 4.5 mg/kg (22). The oral bioavailability was found to be about 87% but the C_{max} values were much lower (C_{max} of 0.062 and 0.046 μmol/L, respectively, after intravenous and oral administrations of 4.5 mg/kg than those reported by Dai and colleagues; refs. 21, 22). The half-life of WA was not affected by route of administration (22). However, much higher levels of WA were reported in tissues including stomach (~5.7 μmol/L), intestine (~2.8 μmol/L), heart (~3.8 μmol/L), liver (~2.0 μmol/L), lung (~3.8 μmol/L), kidney (~3.6 μmol/L), and spleen (~2.1 μmol/L) than in the plasma following a single oral administration of 4.5 mg/kg (22). The half-life of WA in tissues varied between 0.61 (spleen) and 3.21 hours (intestine; ref. 22). The reasons for the discrepancy in results concerning oral bioavailability and C_{max} values from the two rat studies are unclear and require further investigation. Finally, safety and pharmacokinetics of WA were determined in patients with advanced stage high-grade osteosarcoma in a phase I clinical dose escalation study (classical 3 + 3 design in 10 male and 3 female patients) by using liquid chromatography. This study used the root extract of *Withania somnifera* standardized to contain 4.5% of WA (w/w; ref. 23). The dosing regimen reflected daily WA intake of 72, 108, 144, and 216 mg, respectively (23). The authors concluded that WA was well tolerated but it was not detectable in the plasma of any patient (23). However, additional pharmacokinetic studies with pure WA administration and use of more sensitive analytical techniques (LC/MS-MS) and determination of levels in tissue of interest may reveal whether the oral bioavailability of WA is really very low in humans compared with rodents.

In Vivo Studies on WA in Preclinical Models of Breast Cancer

Inhibitory effects of WA in a therapeutic setting

The *in vivo* efficacy studies with WA in preclinical models of breast cancer in a therapy or chemoprevention setting are summarized in **Table 2**. The term chemoprevention was introduced by Dr. Michael B. Sporn signifying "use of pharmacologic agents to impede, arrest, or reverse carcinogenesis at its earliest stages with pharmacologic agents" (24). This definition is now expanded to include natural or synthetic small molecules, dietary or medicinal plant extracts, or biologics (vaccine) for chemoprevention of cancer. In a xenograft study, MDA-MB-231 cells were subcutaneously or orthotopically implanted in female athymic nude mice, and the mice were injected intraperitoneally with either vehicle (10% DMSO, 40% cremophor-EL, and 50% PBS) or the same vehicle containing

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Table 2. *In vivo* efficacy studies with WA in preclinical models of breast cancer.

Experimental model	Dose	Route	Outcome	Ref.
MDA-MB-231 xenograft	4 mg/kg	i.p.	Inhibition of tumor growth (therapy)	(15)
4T1 xenograft	2, 4 mg/kg	i.p.	Inhibition of tumor growth and metastasis (therapy)	(18)
MNU-rat model	4,8 mg/kg	i.p.	Inhibition of tumor incidence (chemoprevention)	(20)
MDA-MB-231 xenograft	4 mg/kg	i.p.	Inhibition of tumor growth (therapy)	(25)
MDA-MB-231 and SUM159 xenograft	100 μg	i.p.	Inhibition of Notch2 knockdown-promoted tumor growth (therapy)	(26)
MDA-MB-231 xenograft	5–20 mg/kg	i.p.	Inhibition of tumor growth (therapy)	(27)
MMTV-*neu* model	100 μg	i.p.	Inhibition of tumor progression (chemoprevention)	(32)
MMTV-*neu* model	100 μg	i.p.	Inhibition of breast cancer stem cells (chemoprevention)	(41)
MDA-MB-231 xenograft	1–8 mg/kg	i.p.	Inhibition of tumor growth and metastasis (therapy)	(42)

Abbreviation: i.p., intraperitoneal.

4 mg WA/kg body weight for 2.5 weeks (15). The average tumor volume in control mice was higher by approximately 1.8-fold compared with WA-treated mice ($P < 0.05$; ref. 15). In another study, the *in vivo* growth of subcutaneously injected MDA-MB-231 cells was inhibited by about 65% as reflected by tumor weight (25). The *in vivo* growth inhibitory effect of WA against genetically modified MDA-MB-231 cells with stable knockdown of the Notch2 protein was also demonstrated (26). Using orthotopic 4T1 mouse mammary cancer model, Thaiparambil and colleagues (18) showed antitumor activity of WA at 2 and 4 mg/kg doses administered by intraperitoneal injection every other day for 30 days. The *in vivo* efficacy of WA in MDA-MB-231 xenograft model was also demonstrated by Liu and colleagues (27).

Breast cancer is a heterogeneous disease that is broadly grouped into major subtypes including, luminal-type, basal-like, HER2-enriched, and normal-like (28). Therefore, the obvious gaps in knowledge regarding *in vivo* cancer therapeutic effects of WA include determination of: (i) whether oral administration of WA inhibits the growth of breast cancer cells, and (ii) whether *in vivo* growth inhibitory effect of WA extends beyond basal-like MDA-MB-231 and 4T1 cells. Further research is necessary to systematically address these important questions.

Inhibitory effects of WA in a chemoprevention setting

Chemoprevention, especially using nontoxic phytochemicals from dietary or medicinal plants such as WA, represents a sensible strategy for decreasing the death and suffering from cancer including breast cancer. Breast cancer is one of the few malignancies for which clinically successful interventions for chemoprevention are available including selective ER modulators like tamoxifen (Nolvadex) and raloxifene (Evista) and aromatase inhibitors such as exemestane (Aromasin) for luminal-type subtype of the disease (29–31). However, a chemopreventive intervention for nonluminal-type breast cancers is still a clinically unmet need. Chemopreventive efficacy of WA has been demonstrated in rodent models representative of two different subtypes, including HER2-driven breast cancer in a mouse model (MMTV-*neu* transgenic mice) and luminal-type breast cancer induced by MNU, a chemical carcinogen (20, 32). In both these models, WA was administered by intraperitoneal route (20, 32). Nevertheless, WA administration significantly inhibited burden and/or incidence of breast cancer in both models (20, 32). In the MMTV-*neu* model, the incidence and burden (macroscopic tumor weight or microscopic tumor area) were scored in female mice after 28 weeks of treatment with 100 μg WA/mouse (about 4 mg/kg body weight for a 25 g mouse), three times/week or vehicle (32). The overall incidence of mammary cancer was not affected by WA administration in the MMTV-*neu* mice (32). However, the mean tumor weight in the WA treatment group was lower by 50% in comparison with the control group with a P value of 0.03 by two-sided Student t test (32). Microscopic examination of the hematoxylin and eosin–stained mammary gland sections also showed a significant decrease in the area (burden) of ductal carcinoma *in situ* and papillary tumor lesions as well as invasive carcinoma in the WA group compared with the control group (32). The mean area of invasive carcinoma, for example, was lower by 95.14% in the WA treatment group compared with the control group (32).

The MNU-induced breast cancer in rats is a widely used model in cancer chemoprevention research. A single MNU injection causes highly reproducible breast tumor development with high incidence in female rats (33). The mammary tumors from this model share histologic similarities with human disease (34). Moreover, the gene expression profiling of breast tumors from the MNU-rat model reveals significant molecular overlap with luminal-type human mammary cancers (35). Preclinical efficacy data using the MNU-rat model were critical for clinical development of tamoxifen and aromatase inhibitor like vorozole (36, 37). We used this model to determine chemopreventive efficacy of WA (20). WA (4 or 8 mg/kg body weight by intraperitoneal route) was administered five times/week for 10 weeks starting 1 week after MNU injection (20). WA administration resulted in a significant decrease in mammary tumor incidence at both doses (20). The tumor multiplicity (average number of tumors/rat) as well as tumor weight were also lower in the WA treatment group compared with vehicle-treated control rats (20). As an example, the wet tumor weight in the 8 mg/kg group was lower by about 68% when compared with control rats (20). Collectively, these studies provided preclinical evidence for chemoprevention of two different subtypes of breast cancer with WA

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administration (20, 32). However, the chemopreventive efficacy of WA against basal-like breast cancers is yet to be determined.

Inhibitory effects of WA on breast cancer stem-like cells *in vivo*

The breast cancer stem-like cells (bCSC), which were first identified by Al-Hajj and colleagues (38), are believed to be responsible not only for breast cancer initiation and progression but also for treatment failure (39, 40). Therefore, it is only logical to develop strategies for elimination of both therapy-sensitive tumor cells constituting bulk of the tumor mass and bCSC to achieve maximal chemopreventive or therapeutic response. The bCSCs are characterized by their ability to form mammospheres and express high aldehyde dehydrogenase 1 (ALDH1) activity. Flow cytometric determination of $CD44^{high}/CD24^{low}$/epithelial-specific antigen-positive (ESA^{+}) population is another technique for quantitation of bCSC fraction. A study from our laboratory showed that WA concentrations ranging from 0.25 to 1 μmol/L significantly inhibited first- and second-generation mammosphere frequency, ALDH1 activity, and $CD44^{high}/CD24^{low}/ESA^{+}$ population in luminal-type (MCF-7) and triple-negative (SUM159) human breast cancer cell lines (41). We also used freshly harvested tumors from control and WA-treated MMTV-*neu* mice (0.1 mg WA by intraperitoneal route, three times/week) to demonstrate a 44% reduction in ALDH1 activity in tumors of WA-treated mice compared with control (41). The WA-mediated decrease in ALDH1 activity was also found in the MNU-rat tumors when compared with control (20). However, it is still unknown whether the bCSC fraction can be suppressed by oral administration of WA. Also, further studies are needed to determine whether bCSC fraction in HER2-enriched cells like SK-BR-3 is sensitive to inhibition by WA.

Inhibitory effects of WA on metastasis

The antimetastatic effect of WA has been reported in mouse models. In one such study, pulmonary metastasis induced by orthotopic injection of 4T1 mouse mammary cancer cells in female Balb/C mice was inhibited significantly by intraperitoneal injection of WA every other day for 30 days (18). The antimetastatic effect of WA was evident at a dose as low as 0.1 mg/kg body weight with about 30% reduction in the number of pulmonary metastatic nodules compared with vehicle-treated control mice (18). More than 70% decrease in pulmonary metastatic nodules was observed at the 4 mg WA/kg body weight (18). In another study, the same group of investigators demonstrated inhibition of pulmonary metastasis induced by mammary fat pad injection of 4T1 by oral administration of ethanol extract of *Withania somnifera* standardized for WA (1, 4, and 8 mg/kg body weight three times a week for 4 weeks; ref. 42). Mice were also treated with WA (1, 4, and 8 mg/kg, intraperitoneal three times/week) to determine whether this small molecule is responsible for antimetastatic effect of *Withania somnifera* (42). Both WA and the ethanol extract of *Withania somnifera* inhibited pulmonary metastasis multiplicity (42). The ethanol extract of *Withania somnifera* exhibited a slightly higher efficacy than WA but only at the 8 mg/kg dose (42). The MMTV-*neu* mice also develop spontaneous pulmonary metastasis. The incidence of pulmonary metastasis was decreased by about 73% upon intraperitoneal administration of 0.1 mg WA/mouse three times/week (32). Collectively, these studies demonstrated antipulmonary metastatic potential of WA.

Metastatic spread to distant organs is the primary cause of morbidity and mortality in subjects with breast cancer. Metastasis in patients with breast cancer may be observed in the bone, lung, liver, and brain, but skeleton is the most preferred site for colonization in each subtype of the disease (ranges between 43% and 71%) when compared with other sites (8%–47%; ref. 43). In future, it would be worthwhile to determine whether the antimetastatic activity of WA extends beyond pulmonary metastasis.

Cancer-relevant Cellular Processes (Cancer Hallmarks) Affected by WA Treatment

The original six hallmarks of cancer included self-sufficiency in growth signals, insensitivity to antigrowth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis, and evasion of apoptosis (44). This list has now been expanded to include new hallmarks like avoiding immune destruction, tumor promoting inflammation, genome instability and mutation, and deregulating cellular energetics (45). Studies have revealed inhibitory effect of WA on many of these cancer hallmarks in breast cancer as summarized in **Fig. 1A** and briefly discussed below.

Inhibition of breast cancer proliferation and cell-cycle arrest by WA treatment

Table 3 summarizes the results of cellular studies on biological effects of WA and includes information on cell lines and WA concentrations used for comparison. Cell viability inhibition by WA in cellular models of breast cancer was initially demonstrated by us using MCF-7 and MDA-MB-231 cells with an IC_{50} between 1.5 and 2.0 μmol/L following 24-hour treatment (15). Since then, several reports have not only confirmed our findings but also evaluated the antiproliferative effect of WA in other breast cancer cell lines including 4T1 (basal), SUM159 (basal), SK-BR-3 (HER2-enriched), T47D (luminal A), Hs578T (basal), and BT474 (luminal B; refs. 18, 25, 46–49). Estrogen-stimulated proliferation of MCF-7 cells was also inhibited significantly in the presence of WA (50). Anticancer effect of WA in the MDA-MB-231 xenograft model, but not in MMTV-*neu* or MNU-rat models, was associated with suppression of Ki-67 or proliferating cell nuclear antigen expression (15, 20, 25, 32).

WA treatment resulted in irreversible G_2–M-phase cell-cycle arrest in MCF-7 and MDA-MB-231 cells that was accompanied by a decrease in levels of key cell-cycle regulators, including

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Table 3. Effective dose range of WA for biological effects in breast cancer cell lines.

Dose of WA (μmol/L)	Cell line	Biological effect	Ref.
0.5–5	MCF-7, MDA-MB-231	Inhibition of growth and induction of apoptosis	(15)
0.027–2	MDA-MB-231	Antiinvasive activity	(18)
5.95	MDA-MB-231	Inhibition of NF-κB and AP-1 Fra-1 transcription factor	(97)
2–4	MCF-7, MDA-MB-231	Inhibition of STAT3 signaling	(82)
1.25–2.5	MCF-7, T47-D, ERα overexpressing MDA-MB-231	Inhibition of ERα signaling	(50)
2.5–5	MCF-7, MDA-MB-231	Induction of ROS-mediated apoptosis	(64)
0.5–5	MCF-7	Downregulation of ERα signaling	(52)
1–4	MCF-7, MDA-MB-231, MDA-MB-468	Activation of Notch2 and Notch4	(58)
0.25–2.5	MDA-MB-231, BT-20	Downregulation of BRCA1 and HSF1	(65)
2.5–5	MCF-7, MDA-MB-231	Induction of apoptosis	(66)
2	MCF-7, MDA-MB-231	Induction of autophagy	(92)
1–5	MCF-7, SUM159	Alteration of MAPK	(67)
2–4	MCF-7, MDA-MB-231	Reversal of EMT	(59)
2–4	MCF-7, SK-BR-3, SUM159	Inhibition of growth arrest associated with downregulation and covalent binding of β-tubulin	(53)
0.7	MDA-MB-231	Antiproliferative, antimetastatic, and epigenetic mode of action	(47)
5	MCF-7, MDA-MB-231	Activation of ERK/RSK to CHOP/Elk1 to DR5 signaling	(25)
0.25–1	MCF-7, SUM159	Inhibition of breast cancer stemness	(41)
1–5	MDA-MB-231	Synergistic apoptotic effect with TNFα	(48)
2–4	MCF-7, MDA-MB-361	Enhanced apoptotic effect with short form RON	(98)
10	SK-BR-3, BT474, ERBB2 overexpressing MCF-7	Enhanced antiproliferative activity by targeting ERBB2/ERBB3	(49)
1–2	MDA-MB-231	Augmented antitumor activity by macrophage	(99)
4	MCF-7, MDA-MB-231	Induction of ROS-mediated paraptosis	(68)
0.7	MCF-7, MDA-MB-231	Silencing of many tumor-promoting genes by DNA methylation	(100)
4	MCF-7, MDA-MB-231	Induction of impaired autophagy	(101)
10	231MFP	Targeting of C377 of PPP2R1A and activation of PP2A activity related to antiproliferative effect	(96)
0.5–10	MCF-7, SK-BR-3, Pin1 (WT and mutant) overexpressing MCF-7	Inhibition of Pin1, covalent binding to Cys-113 of Pin1, and alteration of WA-mediated mitotic arrest and apoptosis by Pin1	(54)
2–4	MCF-7, MDA-MB-231	Inhibition of mitochondrial dynamics associated with apoptosis induction	(70)
2.5–5	MCF-7, MDA-MB-231, MDA-MB-468, SUM149, SUM159	Inhibition of lysosomal activity leading to energy insufficiency and subsequent growth suppression and apoptosis induction	(93)

Abbreviation: WT, wild-type.

cyclin-dependent kinase 1 (Cdk1), cell division cycle 25C (Cdc25C), and/or Cdc25B proteins leading to accumulation of tyrosine 15 phosphorylated (inactive) Cdk1 (51). Overexpression of the Cdc25C protein resulted in partial but statistically significant protection against WA-mediated G_2–M-phase cell-cycle arrest in MDA-MB-231 cells (51). The G_2–M-phase arrest from WA treatment was confirmed by other investigators (52). Another study showed mitotic arrest following WA treatment in MCF-7, SUM159, and SK-BR-3 cells that was associated with a decrease in protein levels of β-tubulin (53). Naturally occurring C6, C7-epoxy analogues of WA (withanone and withanolide A) failed to cause mitotic arrest in these cells (53). The nontumorigenic normal mammary epithelial cell line MCF-10A was more resistant to mitotic arrest by WA when compared with breast cancer cells showing cancer cell–selective mitotic arrest by this agent (53). WA treatment also led to disruption of spindle morphology (53). Mechanism underlying G_2–M-phase arrest by WA seems complex and may involve additional regulators as demonstrated for peptidyl-prolyl *cis/trans* isomerase 1 (Pin1; ref. 54). WA was shown to downregulate Pin1 and its ectopic expression attenuated G_2-phase and/or mitotic arrest resulting from WA in MCF-7 and SK-BR-3 cells (54). WA-induced apoptosis was increased by Pin1 overexpression in MCF-7 cells but not in the SK-BR-3 cell line. Furthermore, WA-mediated chemoprevention of breast cancer in MMTV-*neu* and MNU-rat models was associated with accumulation of mitotic cells as revealed by increased Ser10 phosphorylation of histone H3 *in vivo* (20). Collectively, these studies suggest that G_2–M-phase cell-cycle arrest may be an important mechanism in antiproliferative effect of WA in human breast cancer cells (20, 32, 51–54).

Inhibition of angiogenesis, cell migration and invasion, and epithelial-to-mesenchymal transition by WA treatment in breast cancer cells

Angiogenesis, cell migration and invasion, and epithelial-to-mesenchymal transition (EMT) are critical steps in tumor metastasis (55–57). Published reports have established inhibitory effect of WA on all these prometastatic pathways. Thaiparambil and colleagues (18) were the first to demonstrate

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inhibition of MDA-MB-231 cell invasion by *in vitro* wound healing and Matrigel invasion assays (18). Interestingly, inhibition of MDA-MB-231 cell migration in the presence of WA was evident at noncytotoxic and nonapoptotic doses as low as 27 nmol/L (18). Imaging studies revealed promotion of perinuclear vimentin accumulation followed by rapid vimentin depolymerization after treatment with WA in breast cancer cells, which was accompanied by Ser56 phosphorylation of vimentin (18). Vimentin protein is one of the critical proteins in EMT (56). In another study, WA treatment was shown to cause activation of Notch2 and Notch4 transcription factors, but a decrease in levels of both transmembrane and cleaved form of Notch1 (58). Knockdown of both Notch2 and Notch4 in MDA-MB-231 and MDA-MB-468 cells augmented WA-mediated inhibition of cell migration (58). This study suggested an undesirable effect where Notch2 and Notch4 activation impeded inhibitory effect of WA on breast cancer cell migration (58). A single-cell collagen invasion assay was also used to demonstrate inhibitory effect of WA on MDA-MB-231 cell invasion ability (47). Gene expression profiling in this study revealed a decrease in expression of several extracellular matrix–degrading proteases (*uPA, PLAT,* and *ADAM8*), cell adhesion molecules (integrins and laminins), and certain proinflammatory mediators (*TNFSF12, IL6, ANGPTL2,* and *CSF1R*) by WA treatment (47). It is still unknown whether these gene expression changes affected by WA treatment are applicable to cell lines other than MDA-MB-231.

An experimental model of EMT following treatment of nontumorigenic MCF-10A cells with TNFα and TGFβ was used to demonstrate inhibitory effect of WA (59). Inhibition of experimental EMT and cell migration by WA treatment was partially reversed by combined TNFα and TGFβ treatments (59). Downregulation of E-cadherin is one of the hallmarks of EMT phenotype and breast cancer cells exposed to WA exhibited sustained (MCF-7) or transient (MDA-MB-231) induction of E-cadherin protein expression (56, 59). Furthermore, the level of vimentin protein was significantly lower in the MDA-MB-231 xenografts and MMTV-*neu* tumors from WA-treated mice compared with corresponding controls (59). Finally, human umbilical vein endothelial cells were used to determine antiangiogenic effect of WA (60). However, the number of CD31-positive blood vessels, a marker of neoangiogenesis, was not significantly altered by WA administration *in vivo* in either MMTV-*neu* or MNU-rat models (20, 32).

Modulation of DNA damage response by WA in breast cancer cells

Ataxia telangiectasia and Rad3-related (ATR)-checkpoint kinase 1 (CHK1) signaling plays an important role in the DNA damage response pathway for maintenance of the genomic integrity (61, 62). The ATR kinase is activated by replication stress during cell division or genotoxic insult and functions at the S- and G_2-phase of the cell cycle (61, 62). Human breast cancer cells (MCF-7, MDA-MB-231, and SUM159) treated with WA showed suppression of protein level as well as phosphorylation of ATR and CHK1 due to both transcriptional and posttranscriptional mechanisms (63). Forced expression of CHK1 abolished the WA-mediated G_2–M-phase arrest but increased Ser10 phosphorylation of histone H3 (63). A trend for a decrease in the protein level of ATR was found in the mammary tumors of WA-treated MMTV-*neu* mice but the difference was not significant (63). A clinically relevant observation of this study was sensitization of MDA-MB-231 and SUM159 cells to growth inhibition by cisplatin, but the *in vivo* effect of this potential combination regimen is yet to be determined (63). Additional studies are also needed to determine whether WA treatment affects other DNA damage response pathways.

Induction of apoptosis by WA treatment in breast cancer cells

WA is best studied for its proapoptotic effect (15, 25, 27, 52, 64–70). We were the first to report apoptosis induction by WA in MCF-7 and MDA-MB-231 cells by Western blotting for PARP cleavage and quantitation of DNA fragment release into the cytosol (15). The WA-mediated *in vivo* growth inhibition of MDA-MB-231 xenograft was accompanied by apoptosis induction as revealed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (15). The WA-mediated chemoprevention of breast cancer in both MMTV-*neu* and MNU-rat models was also associated with increased number of TUNEL-positive apoptotic cells (20, 32). Since then, proapoptotic effect of WA has been confirmed by other investigators in cell lines other than MCF-7 and MDA-MB-231 (15, 25, 27, 52, 64–70). Mechanistically, generation of reactive oxygen species (ROS) is implicated in proapoptotic effect of WA (27, 64). WA-mediated inhibition of oxidative phosphorylation (OXPHOS) at complex III of the mitochondrial electron transport chain was responsible for ROS generation (64). The ROS generation by WA is specific for cancer cells as this was observed in MDA-MB-231 and MCF-7 cells, but not in a normal human mammary epithelial cell line HMEC (64). The HMEC cells were also resistant to proapoptotic effect of WA (64). Overexpression of Cu, Zn-superoxide dismutase (SOD1) in MDA-MB-231 or MCF-7 cells or depletion of mitochondrial DNA (Rho-0 cells) also elicited resistance to WA-induced ROS production, collapse of mitochondrial membrane potential, and apoptosis (64). A critical role for ROS-dependent activation of Bax and/or Bak has also been suggested as a mechanism for WA-induced apoptosis (64). However, the mechanism by which WA inhibits complex III activity is still not completely understood, although we have reported a decrease in assembly of complex III in MCF-7 and SUM159 cells, but not in MDA-MB-231, after treatment with WA as determined by native blue gel electrophoresis (70). WA treatment also inhibited chemically induced mitochondrial fusion in breast cancer cells associated with downregulation of proteins involved in fusion process (mitofusin1, mitofusin2, and full-length optic atrophy protein 1; OPA1). The level of mitochondrial fission–regulating protein, dynamin-related

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protein 1 (DRP1), was decreased by WA exposure and its deficiency as well as OPA1 knockdown attenuated apoptotic effect of WA (70). One study suggested induction of paraptosis, a form of cell death morphologically distinct from apoptosis or autophagy, by WA treatment (68) but the mechanism underlying this response or its *in vivo* relevance is yet to be determined.

Inhibition of aerobic glycolysis by WA treatment in breast cancer cells

Metabolic reprogramming including increased glycolysis in tumor cells was added to the list of cancer hallmarks in the year 2011, even though this phenomenon was first described in the 50s by Dr. Otto Warburg (45, 71). Normal cells derive ATP from OXPHOS, whereas cancer cells become addicted to aerobic glycolysis to meet energy demand (71, 72). Through unbiassed global metabolomics, we reported a decrease in levels of several glycolysis and tricarboxylic acid cycle intermediates in the plasma and/or mammary tumor tissue of WA-treated MMTV-*neu* mice when compared with control mice (32). A cartoon summarizing WA-mediated suppression of the plasma and/or mammary tumor levels of glycolysis and tricarboxylic acid cycle intermediates (highlighted in red color) from MMTV-*neu* and MNU-rat models is shown in **Fig. 2**. Expression of many enzyme proteins related to glycolysis and tricarboxylic acid cycle was also lower in the tumors of WA-treated MMTV-*neu* mice than those of control mice (highlighted in green in **Fig. 2**; ref. 32). The WA-mediated suppression of glycolysis is not unique to the MMTV-*neu* model as a decrease in plasma lactate level was also observed in the MNU-rat model (20, 32). Furthermore, WA administration resulted in suppression of glutamine and acetyl-CoA levels (20, 32). Acetyl-CoA is the building block of fatty acid synthesis. Increased fatty acid synthesis or increased cholesterol uptake has been reported in HER2-enriched or triple-negative breast cancers (reviewed in ref. 73). Thus, it is possible that WA treatment inhibits fatty acid synthesis in breast cancer cells, but additional work is necessary to test this hypothesis. In addition, the molecular basis for metabolic inhibition by WA is yet to be elucidated.

It is interesting to note that WA treatment inhibits OXPHOS in MCF-7 and MDA-MB-231 cells (64). Seahorse flux analysis revealed that basal oxygen consumption rate (OCR), which is a measure of OXPHOS, was relatively higher in the MCF-7 cell line when compared with MDA-MB-231 (64). However, both cells exhibited a statistically significant decrease in basal OCR following 4-hour exposure to 2.5 and 5 μmol/L WA (64). The inhibitory effect of WA on basal OXPHOS was relatively more pronounced in the MDA-MB-231 cell line compared with MCF-7 (64). WA treatment resulted in a statistically significant decrease in reserve OXPHOS in MDA-MB-231 and MCF-7 cells especially at the 5 μmol/L dose (64). However, it is unclear whether WA treatment inhibits OXPHOS in HER2-enriched breast cancer cell lines *in vitro* or in breast cancer cells of different subtypes *in vivo*. We also know that WA targets complex III of the electron transport chain to disrupt electron flow that may explain the ROS production by this phytochemical.

Immune modulatory effect of WA

Myeloid-derived suppressor cells (MDSC) play an important role in tumor promotion through inhibition of T-cell function and cross-talk with tumor-associated macrophages (74). One of the mechanisms of evasion of tumor immunity by MDSC is the result of their cross-talk with tumor-associated macrophages for increased production of IL10 (75). WA treatment was shown to inhibit MDSC and IL10 production (76). Macrophages secrete IL6 and TNFα that enhance accumulation and function of MDSC (74). Secretion of IL6 and TNFα by macrophages was also inhibited by WA treatment (76). Oral administration of WA (1–8 mg/kg body weight, three times/week) to 4T1 mouse mammary tumor–bearing mice resulted in suppression of granulocytic MDSC (76). In another study, WA treatment inhibited mitogen-stimulated secretion of IL2, IL4, IL6, and IFNγ in $CD8^+$ and $CD4^+$ T cells (77). Immune modulatory effects of *Withania somnifera* extract have also been reported that may be attributable to WA (78, 79). However, the *in vivo* immune modulatory effects of WA in preclinical models of different subtypes of breast cancer are yet to be determined.

Molecular Targets of WA in Breast Cancer Cells

As described below and summarized in **Fig. 1B**, WA targets multiple transcription factors, receptors, and kinases to elicit its anticancer responses, including apoptosis induction and inhibition of cell proliferation, cell migration/invasion, and self-renewal of bCSC.

FOXO3a

FOXO3a, a member of the forkhead box transcription factors, was the first identified mechanistic target of WA in breast cancer cells (15). FOXO3a is widely implicated in different solid tumors including breast cancer, and existing evidence indicates a tumor suppressor function for this transcription factor (80). FOXO3a knockdown in MCF-7 cells resulted in a partial but meaningful protection against WA-mediated apoptosis involving its downstream proapoptotic target Bim (15).

STAT3

Overexpression and constitutive activation of STAT3 has been implicated in the progression, proliferation, metastasis, and chemoresistance of breast cancer (81). STAT3 is another transcription factor whose activity is suppressed by WA treatment in breast cancer cells (82). WA treatment was shown to inhibit constitutive and/or IL6-inducible activation of STAT3 in MCF-7 and MDA-MB-231 cells as well as phosphorylation of its upstream regulator JAK2 (82). Treatment of MDA-MB-231 or

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MCF-7 cells with WA also led to suppression of (i) transcriptional activity of STAT3 with or without IL6 stimulation; (ii) dimerization of STAT3 at least in MDA-MB-231 cells; and (iii) nuclear translocation of phosphorylated STAT3 in both cells (82). The IL6-mediated activation of STAT3 conferred a partial protection against cell invasion inhibition by WA in MDA-MB-231 cells (82).

ERα

ERα is very well-studied for its protumorigenic function in breast cancer (83). Growth inhibition and apoptosis induction by WA treatment in MCF-7 cells were significantly attenuated by ERα ligand 17β-estradiol (E2; ref. 50). MCF-7 cells exposed to WA exhibited decreased protein levels of ERα (but not ERβ) and ERα-regulated gene product pS2, and this effect was also markedly attenuated by E2 (50). Overexpression of ERα in the MDA-MB-231 cell line conferred partial but statistically significant protection against WA-mediated apoptosis, but not the G_2–M-phase cell-cycle arrest (50). Downregulation of ERα protein expression in MCF-7 cells following WA treatment was confirmed by another group of investigators (52).

p53

p53, which is known to regulate cell cycle and apoptosis by different stimuli, is a well-known tumor suppressor (84). WA treatment caused induction as well as increased Ser15 phosphorylation of p53 (activation) in MCF-7 cells, although RNAi of this tumor suppressor conferred only a modest protection against WA-induced apoptosis at least in this cell line (50).

Receptors and transcription factors linked to bCSC maintenance

Expression profiling for stemness-related genes revealed (*Oct4, SOX-2*, and *Nanog*) suppression of only *SOX-2* mRNA after 24-hour WA treatment in MCF-7 cells and downregulation of *Oct4*, *SOX-2*, and *Nanog* expression at the 72-hour timepoint in SUM159 cells (41). However, the precise function of these stemness-related genes in WA-mediated inhibition of bCSC is yet to be determined. Urokinase-type plasminogen activator receptor (uPAR) overexpression alone is sufficient to drive stemness in MCF-7 cells (85). Overexpression of uPAR conferred partial but significant protection against bCSC inhibition by WA. Interestingly, WA treatment resulted in induction of Krüppel-like factor 4 (KLF4) expression, which was shown to be required for maintenance of bCSC and mammary cancer cell migration and invasion (86), in MCF-7 and SUM159 cells, and its knockdown by KLF4-targeted siRNA transfection augmented bCSC inhibition by WA (41). The Hedgehog pathway is implicated in hormone receptor–positive and triple-negative breast cancers (87). One study identified WA as an inhibitor of the Hedgehog pathway, but experiments to determine the functional relevance of this finding were not conducted (88).

Kinases

Effect of WA has been studied on ERK, p38 MAPK, and c-Jun N-terminal kinases (JNK) using breast cancer cells (all three kinases were activated by WA treatment in both cells; ref. 67). Overexpression of an antioxidative enzyme protein (manganese-superoxide dismutase) was partially protective against WA-mediated hyperphosphorylation (activation) of ERK, but not JNK or p38 MAPK (67). Apoptosis induction by WA treatment was significantly augmented by pharmacologic inhibition of ERK and p38 MAPK in MCF-7 cells (67). On the other hand, WA-mediated apoptosis in MCF-7 cells was partially attenuated by JNK inhibition (67). Inhibition of ERK or JNK had no meaningful impact on WA-induced apoptosis in the SUM159 cell line. However, the effect of WA on these pathways in HER2-enriched breast cancer cells or normal mammary cells is yet to be determined. In another study, a rather high concentration of WA (10 μmol/L) was used to show downregulation of ERBB2 (49). Overexpression of ERBB2 sensitized breast cancer cells to WA (49). Downstream effects included suppression of AKT by WA treatment (49). Increased phosphorylation of ribosomal S6 kinase (RSK) was also reported in breast cancer cells following WA treatment (25). RSK activation by WA led to activation of ETS-like transcription factor 1 and C-EBP homologous protein kinase pathways and subsequent upregulation of death receptor 5 to cause apoptotic cell death (25).

Inhibitor of apoptosis family members

The inhibitor of apoptosis (IAP) family protein members regulate apoptosis by inhibiting caspases (89, 90). Treatment with WA resulted in downregulation of XIAP, cIAP-2, and survivin protein levels in MCF-7 and MDA-MB-231 cells (66). Apoptosis induction by WA was attenuated by overexpression of XIAP, survivin, and cIAP-2 in both cell lines (66). Interestingly, the inhibition of MDA-MB-231 xenograft growth by WA administration was associated with statistically significant downregulation of only survivin protein expression (66). The *in vivo* role of the IAP family members in breast cancer prevention by WA is yet to be elucidated.

Autophagy regulation proteins

The autophagy is an evolutionarily conserved process for recycling of cellular macromolecules as well as organelles like mitochondria (91). This process is tightly regulated by the members of the autophagy-related gene (ATG) family (91). Our laboratory was the first to demonstrate autophagy induction in MCF-7 and MDA-MB-231 cells following treatment with WA (92). However, this process is not cancer selective as autophagy induction by WA was also observed in the MCF-10A cell line (92). Inhibition of MDA-MB-231 and MCF-7 cell viability resulting from WA treatment was not affected by either pharmacologic suppression of autophagy with 3-methyl adenine or genetic repression of ATG5 (92). Autophagy induction by WA in breast cancer cells was subsequently confirmed by other investigators (27, 93).

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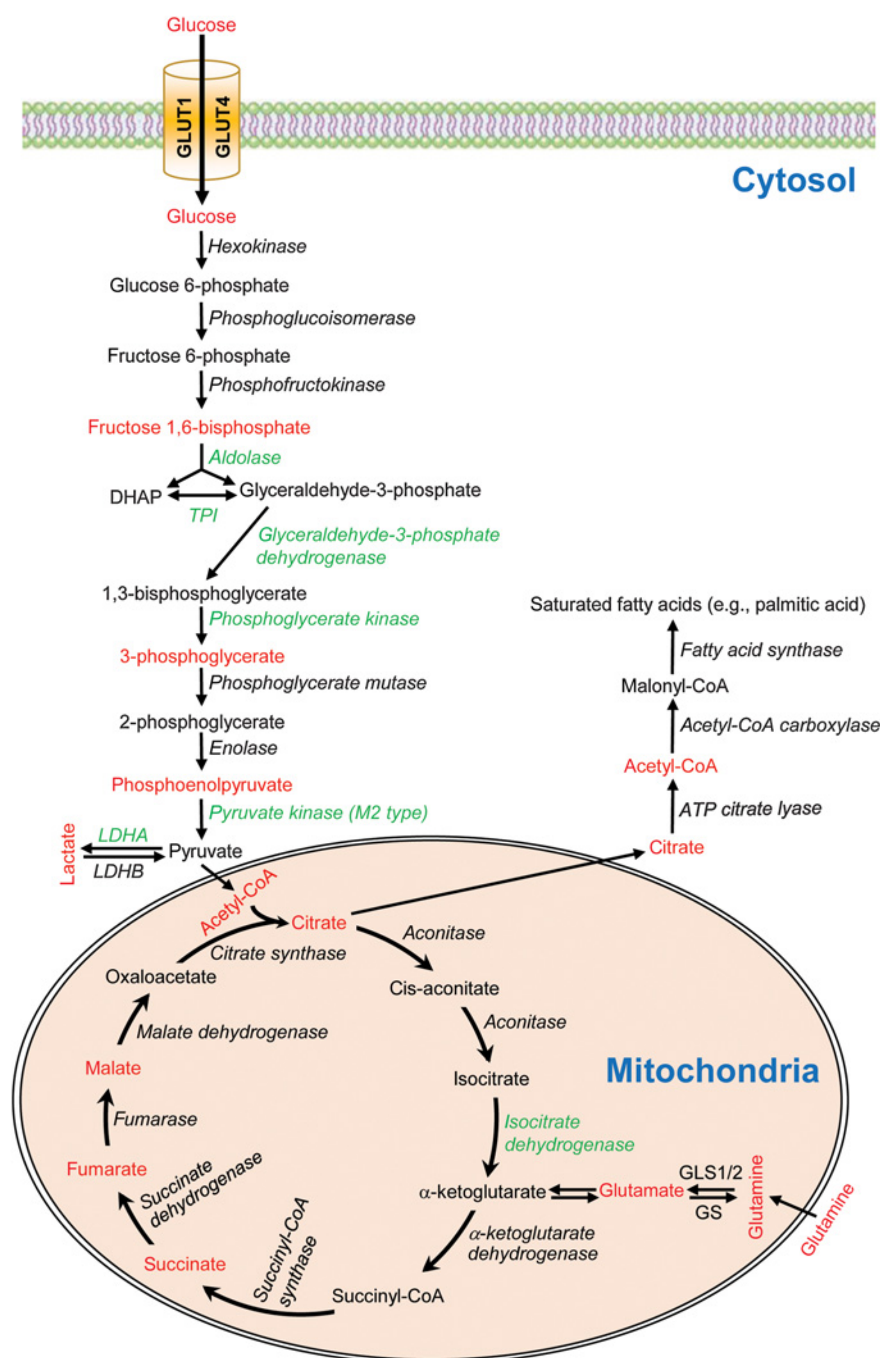

Figure 2.
Inhibitory effects of WA on metabolic intermediates in breast cancer cells. The metabolite levels suppressed by WA are identified by red color, whereas WA-mediated downregulation of proteins is shown in green color.

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Covalent modification of proteins

Because WA is an electrophile (Michael acceptor), it can directly react with cysteine residue(s) in different proteins. Bargagna-Mohan and colleagues (94) were the first to demonstrate modification of cysteine-328 of vimentin protein by WA treatment (5 μmol/L) using endothelial cells. Subsequent work from our own laboratory showed covalent modification of cysteine-303 of β-tubulin following treatment of MCF-7 cells with 2 μmol/L WA (53). In this study, we used NMR to demonstrate that the A-ring enone in WA was highly reactive with cysteamine (a nucleophile) and rapidly succumbed to irreversible nucleophilic addition (53). Molecular docking indicated that the WA-binding pocket was located on the surface of β-tubulin and characterized by a hydrophobic floor, a hydrophobic wall, and a charge-balanced hydrophilic entrance. These results provided novel insights into the mechanism of growth arrest by WA in breast cancer cells (53). Covalent modification of cysteine-179 of IKKβ after treatment of human embryonic kidney HEK293T cells with 5 μmol/L WA was also reported as a mechanism of NF-κB inhibition (95).

Using a synthetic NF-κB-p50 peptide, Gambhir and colleagues (77) reported covalent modification of cysteine-62 residue. More recently, Grossman and colleagues (96) used chemoproteomic platforms to map the proteome-wide cysteine modification by WA. These investigators concluded that cysteine-377 on the regulatory subunit of the tumor-suppressor protein phosphatase 2A was a target of WA at least in the 231MFP cell line, which is a highly aggressive variant of the MDA-MB-231 human breast cancer cell line (96). However, other previously known modifications (e.g., vimentin, β-tubulin, or IKKβ) could not be validated in the study by Grossman and colleagues (96), which also did not rule out the possibility of covalent modification of previously published targets under different experimental conditions. Pin1 protein has two cysteine residues at positions 57 and 113. Molecular docking suggested interaction of WA with cysteine-113 of Pin1 that was confirmed by mass spectrometry (54).

Conclusions, Future Directions, and Gaps in Knowledge

Cancer cell selectivity, oral bioavailability, target tissue bioavailability, safety, and efficacy are desired characteristics for clinical development of a chemopreventive intervention. WA meets all these criteria based on preclinical studies discussed in this article. However, there are still critical knowledge gaps that need to be filled before clinical investigation of WA. First, the kinetics of mammary/tumor tissue bioavailability and clearance of WA is yet to be determined, which is essential for optimization of dosing schedule. Cellular *in vitro* studies indicate sensitivity of basal-like breast cancer cells to WA, but it is important to test whether development of this subtype of breast cancer is prevented by WA administration even though xenograft studies have shown *in vivo* therapeutic response of WA against basal-like human breast cancer cells. Cellular *in vitro* mechanistic studies have largely focused on luminal-type MCF-7 and basal-like MDA-MB-231 cells. Similar studies are needed in HER2-enriched breast cancer cell line like SK-BR-3 to broaden the mechanistic knowledge. Although, cell viability inhibition and mitotic arrest by WA have been reported in luminal, basal, and HER2-enriched breast cancer cells (53). Nevertheless, the preclinical results are quite compelling to advance WA to a phase I trial. A current limitation is that WA is not yet approved by the FDA.

Disclosure of Potential Conflicts of Interest

S.V. Singh reports grants from NIH during the conduct of the study. No potential conflicts of interest were disclosed by the other authors.

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