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A Review on Perspective of Withaferin A as Anticancer Agent

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Abstract— *Withania somnifera (L.) is a native Asian plant that contains a large amount of Withaferin A, sometimes known as WA for short. WA is a potential steroidal lactone with anticancer properties. Ayurvedic medicine still uses the root/leaf extract and formulations of Withania somnifera for the practice of alternative medicine, a member of the Solanaceae family. Withania somnifera's root/leaf extract contains a wide range of compounds, including sitoindosides, alkaloids, and withanolides (WA, withanone, withanolide A, etc.), however WA is primarily responsible for the plant's anticancer properties. Early in the 1970s, the Ehrlich ascites tumor cell model was used to show the anticancer action of WA in vitro. Since then, a great deal of preclinical research has been done to ascertain the cancer therapeutic and chemopreventive effects of WA utilizing cellular and animal models of various malignancies, including breast cancer. The term "chemoprevention" coined by Dr. Michael B. Sporn, refers to the use of pharmaceutical medicines to stop, halt, or reverse carcinogenesis in its early stages. This review provides a concise overview of the published research on the anticancer pharmacology of WA in breast cancer, with particular attention to its pharmacokinetic behavior, in vivo efficacy data in preclinical models in therapeutic and chemoprevention settings, and its known effects on molecular targets and cancer-relevant cellular processes, including growth arrest, autophagy, apoptosis induction, metabolic adaptation, immune function, and growth arrest. There was also discussion of potential information gaps and future research areas that are crucial for the clinical development of WA for breast cancer treatment or chemoprevention.*

Keywords– *Breast cancer, Chemoprevention, Withania somnifera (L.), Withaferin A*

I. INTRODUCTION

Throughout the world, breast cancer is a severe health issue that affects hundreds of thousands of women. In 2020, it is anticipated that over 40,000 women would succumb to breast cancer in the United States alone (Siegel & Miller, 2020). There remains a need for innovative therapeutic and preventative approaches to reduce the disease's deaths and sorrow. Research on the use of medicinal plant extracts or the small molecules they contain as novel approaches to treatment and/or chemoprevention of breast cancer is still ongoing. *Withania somnifera*, a member of the Solanaceae family of plants and popularly known as Ashwagandha or Indian winter cherry, is a fascinating medicinal herb that is the subject of extensive research due to its potential to treat cancer and other illnesses. In India and the surrounding nations, formulations of Ayurvedic, Siddha, and Unani medicine still contain *Withania somnifera* root/leaf extract (Palliyaguru et al., 2017) (Nasimi et al., 2018) (Sarris, 2018) (Srivastav et al., 2017) (Tandon & Yadav, 2020) (Saggam et al., 2020). On ClinicalTrials.gov, there are over 15 clinical trials utilizing *Withania somnifera* extract for various medical diseases. The control of male reproductive systems, neuroprotective potential, relief from stress and anxiety, enhancement of memory and cognitive functions, muscle strength and recovery, and other clinical effects of *Withania somnifera* extract have all been researched (Prospective et al., 2012) (Committee et al., 2013) (Wankhede et al., 2015) (Choudhary et al., 2017). In the US, *Withania somnifera* extract is sold as a nutritional supplement over-the-counter.

The presence of withanolides, alkaloids, and sitoindosides in *Withania somnifera* extract demonstrates its rich diversity of phytochemical makeup. While the anticancer potency of each identified chemical component of the *Withania somnifera* extract is still unknown, the withanolide family member withaferin A has been the subject of the most research for its potential to treat various cancers, including breast cancer (Shohat B, Gitter S, Abraham A, n.d.) (H. Yang et al., 2007) (Srinivasan S, Ranga RS, Burikhanov R, Han SS, n.d.) (Stan et al., 2008) (Vyas & Singh, 2014) (Cai et al., 2014) (Thaiparambil et al., 2011). The extensive literature on the anticancer effects of WA and its pharmacology is compiled in this review, with particular attention to the substance's pharmacokinetic behavior, in vivo efficacy data in preclinical rodent models of breast cancer, and its known effects on molecular targets and cancer-relevant cellular processes, such as the suppression of estrogen receptor-α, signal transducer and activator of transcription 3, cell cycle arrest, and induction of apoptosis and autophagy, metabolic adaptation, and immune function. Knowledge gaps and potential avenues for future study are also covered in order to support the clinical development of WA for breast cancer treatment or chemoprevention.

The pharmacokinetic characteristics of WA

An agent's pharmacokinetic behaviour must be understood in order for it to be developed clinically. This information is essential for preclinical and clinical studies' dosing schedules based on tissue availability and clearance, half-lives, and other factors, as well as for in vitro mechanistic studies (e.g., dose selection based on maximum plasma/serum achievable level to avoid use of supra-pharmacological concentrations). A single intraperitoneal injection of 4 mg/kg body weight of WA produced a maximum plasma concentration (Cmax) of

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approximately 1.8 µmol/L with a half-life of approximately 1.3 hours in female Balb/c mice (Thaiparambil et al., 2011). This investigation yielded an estimated exposure area under the curve value of 1.09 µmol/L / hour (Thaiparambil et al., 2011). Reverse phase liquid chromatography-tandem mass spectrometry (LC/MS/MS) was used to get these results (Thaiparambil et al., 2011). After giving female Swiss albino mice an oral dose of 1000 mg/kg of an aqueous extract of *Withania somnifera* root, pharmacokinetic characteristics for WA were ascertained in a different investigation employing LC/MS/MS (Patil D, Gautam M, Mishra S, Karupothula S, Gairola S, Jadhav S, 2013). After an intraperitoneal injection, the Cmax of WA was found to be higher (18.7 ± 4 ng/mL) with an observed Tmax (time to reach Cmax) of 20 minutes (Patil D, Gautam M, Mishra S, Karupothula S, Gairola S, Jadhav S, 2013). Given that water is not the ideal solvent for extracting WA because it is a hydrophobic molecule, the lower plasma level of WA following oral administration of the aqueous *Withania somnifera* extract makes sense. In more recent times, two distinct teams of researchers have assessed the pharmacokinetics and oral bioavailability of WA in rats, yielding remarkably diverse results (Dai T et. al., 2019) (Wang et al., 2019). The WA levels were measured in both of these trials using the LC/MS/MS technique (Dai T et. al., 2019) (Wang et al., 2019). After oral and intravenous injections of 10 mg/kg and 5 mg/kg, respectively, male Sprague-Dawley rats' plasma had an oral bioavailability of roughly 20%, according to Dai et al (Dai T et. al., 2019). After oral and intravenous doses, the Cmax for WA was approximately 1.3 and 6.5 µmol/L, respectively (Dai T et. al., 2019). In the other trial, Sprague-Dawley rats (sex not stated) were used to assess the pharmacokinetic characteristics of WA following a single intravenous infusion of 4.5 mg/kg or a single oral treatment of 0.5 mg/kg, 1.5 mg/kg, and 4.5 mg/kg (Wang et al., 2019). Oral bioavailability was determined to be approximately 74%, however Cmax values were significantly lower than those reported by Dai et al (Dai T et. al., 2019) (Wang et al., 2019) Cmax of 0.062 µmol/L and 0.046 µmol/L, respectively following intravenous and oral administrations of 4.5 mg/kg. The spleen had a half-life of 0.61 hours, while the intestine had a half-life of 3.21 hours (Wang et al., 2019). More research is necessary to determine the causes of the inconsistent oral bioavailability and Cmax values between the two rat experiments' results. Lastly, using liquid chromatography, the safety and pharmacokinetics of WA were ascertained in patients with advanced stage high-grade osteosarcoma in a phase I clinical dose escalation research (traditional 3 + 3 design in 10 male and 3 female patients). The root extract of *Withania somnifera*, which was standardized to contain 4.5% of WA (w/w), was employed in this study (Pires et al., 2020). The daily WA intake of 72 mg, 108 mg, 144 mg, and 216 mg was included in the dosage schedule (Pires et al., 2020). Although WA was not found in any patient's plasma, the investigators judged that it was well tolerated (Pires et al., 2020). Nevertheless, future pharmacokinetic research using pure WA administration, the application of more exacting analytical methods (LC/MS/MS), and the assessment of levels in relevant tissue may provide insight into whether or not human's oral bioavailability of WA is actually significantly lower than that of rats.

Research on WA in vivo using preclinical models of breast cancer

(A) WA's inhibitory effects in a therapeutic context

Dr. Michael B. Sporn coined the term "chemoprevention," which means "using pharmaceutical agents to impede, arrest, or reverse carcinogenesis at its earliest stages" (Sporn, 1991). This term has been modified to include biologics (vaccines) for cancer chemoprevention, nutritional or medicinal plant extracts, and artificial or natural small molecules. Female athymic nude mice were used in a xenograft study. Subcutaneous or orthotopically implanted MDA-MB-231 cells were given to the mice, and for 2.5 weeks, they received intraperitoneal injections of either vehicle (10% dimethyl sulfoxide, 40% cremophor-EL, and 50% phosphate-buffered saline) or the same vehicle containing 4 mg WA/kg body weight (Stan et al., 2008). Compared to mice treated with WA, the average tumor volume in control mice was approximately 1.8 times greater ($P < 0.05$) (Manuscript & Apoptosis, 2009). Tumor weight indicated that in vivo development of MDA-MB-231 cells administered subcutaneously was suppressed by approximately 65% in a different study (Nagalingam A, Kuppusamy P, Singh SV, Sharma D, 2015). It has also been shown that WA inhibits the proliferation of genetically altered MDA-MB-231 cells with a stable Notch2 protein knockdown in vivo (Hahm et al., 2021). (Thaiparambil et al., 2011) demonstrated the anticancer efficacy of WA at 2 mg/kg and 4 mg/kg dosages provided by intraperitoneal injection every other day for 30 days using an orthotopic 4T1 mouse mammary cancer model. Liu et al. also showed the in vivo effectiveness of WA in the MDA-MB-231 xenograft model (Liu X et. al.,2019). Breast cancer is a diverse condition that can be roughly classified into four main subtypes: basal-like, luminal-type, HER2-enriched, and normal-like (Sørlie et al., 2001). Determining whether oral administration of WA inhibits the growth of breast cancer cells and whether the in vivo growth inhibitory effect of WA extends beyond basal-like MDA-MB-231 and 4T1 cells are thus the obvious gaps in our understanding regarding the in vivo cancer therapeutic effects of WA. In order to thoroughly answer these significant problems, more study is required.

(B) WA's inhibitory actions in a chemoprevention context

Chemoprevention is a reasonable approach to reducing the death and suffering from cancer, particularly breast cancer. This is especially true when employing non-toxic phytochemicals from nutritional or medicinal plants like WA. One of the few cancers for which there are clinically effective chemoprevention interventions is breast cancer. These include aromatase inhibitors, such as exemestane (Aromasin®), for the luminal-type subtype of the disease, and selective estrogen receptor modulators, such as tamoxifen (Nolvadex®) and raloxifene (Evista®) (Fisher B. et. al., 1998) (Cauley et al., 2001) (Goss et al., 2011). A chemopreventive strategy is still lacking in clinical practice for non-luminal breast tumors, nevertheless. The chemopreventive effectiveness of WA has been proven in rodent models that represent two distinct

subtypes: luminal-type breast cancer produced by MNU, a chemical carcinogen, and HER2-driven breast cancer in a mouse model (MMTV-neu transgenic mice) (Samanta et al., 2017) (Hahm et al., 2013). WA was injected intraperitoneally into both of these models (Samanta et al., 2017) (Hahm et al., 2013). However, in both models, WA treatment markedly reduced the burden and/or incidence of breast cancer (Hahm et al., 2013) (Samanta et al., 2017). In the MMTV-neu model, after 28 weeks of therapy with 100 µg WA/mouse (about 4 mg/kg body weight for a 25g mouse), three times/week, or vehicle, the incidence and burden (microscopic tumor weight or microscopic tumor area) were assessed in female mice (Hahm et al., 2013). In MMTV-neu mice, treatment of WA had no effect on the overall incidence of breast cancer (Hahm et al., 2013). Nonetheless, a two-sided Student t test revealed that the mean tumor weight in the WA treatment group was 50% lower than in the control group, with a P value of 0.03 (Hahm et al., 2013). When comparing the WA group to the control group, microscopic analysis of the mammary gland sections stained with hematoxylin and eosin revealed a noteworthy reduction in the area (burden) of ductal carcinoma in situ, papillary tumor lesions, and invasive carcinoma (Hahm et al., 2013). For instance, the WA treatment group's mean area of invasive carcinoma was 95.14% less than the control group's (Hahm et al., 2013).

Rats with MNU-induced breast cancer are a commonly used model in studies on cancer chemoprevention. In female rats, a single MNU injection results in a high rate of very repeatable breast tumor formation (Thompson et al., 1991). The histological characteristics of the breast tumors in this model are comparable to those of human disease (Russo et al., 1990). Furthermore, there is a notable molecular overlap between breast tumors from the MNU-rat model and human mammary malignancies of the luminal type, according to the gene expression profile of these tumors (Chan et.al., 2005). For the clinical development of tamoxifen and aromatase inhibitors like vorozole, preclinical efficacy results utilizing the MNU-rat model were essential (Gottardis et al., 1987) (Lubet et al.,1998). We assessed the chemopreventive effectiveness of WA using this model (Samanta et al., 2017). One week following the MNU injection, WA (4 mg/kg or 8 mg/kg body weight by intraperitoneal method) was given five times a week for ten weeks (Samanta et al., 2017). At both doses, the injection of WA significantly reduced the incidence of breast tumors (Samanta et al., 2017). In addition, the WA treatment group's tumor weight and multiplicity (average number of tumors/rat) were lower than those of the vehicle-treated control rats (Samanta et al., 2017). For instance, compared to control rats, the wet tumor weight in the 8 mg/kg group was approximately 68% smaller (Samanta et al., 2017). When taken as a whole, these investigations offered preclinical support for the use of WA as chemoprevention against two distinct subtypes of breast cancer (Samanta et al., 2017) (Hahm et al., 2013). It is unknown, though, how effective WA is as a chemopreventive treatment for breast cancers that resemble basal-like tumors.

(C) WA's in vivo inhibitory effects on breast cancer stem-like cells (bCSC)

First discovered by Al-Hajj and associates, the bCSCs are thought to be in charge of treatment failure as well as the onset and advancement of breast cancer (Al-hajj et al., 2003) (Marco et al., 2012) (Brien et al., 2011). To obtain the greatest chemopreventive and therapeutic response, it makes sense to create techniques for the eradication of both bCSC and therapy-sensitive tumor cells, which make up the majority of the tumor mass. The capacity to generate mammospheres and exhibit high levels of aldehyde dehydrogenase 1 (ALDH1) activity are two characteristics of bCSCs. A further method for quantifying the percentage of bCSCs is flow cytometric measurement of the $CD44^{high}/CD24^{low}$/epithelial-specific antigen-positive (ESA+) population. In the MNU-rat tumors, the WA-mediated reduction in ALDH1 activity was also observed in contrast to the control (Samanta et al., 2017). Nevertheless, whether or whether oral WA treatment can lower the bCSC fraction is still uncertain. Additionally, more research is required to ascertain whether the bCSC fraction in HER2-enriched cells, such as SK-BR-3, is susceptible to WA inhibition.

(D) WA's inhibitory actions on metastasis

In mouse models, the anti-metastatic action of WA has been documented. In one such study, intraperitoneal injection of WA every other day for 30 days effectively prevented lung metastasis in female Balb/C mice induced by orthotopic injection of 4T1 mouse mammary cancer cells (Thaiparambil et al., 2011). When compared to vehicle-treated control mice, the number of lung metastatic nodules was approximately 30% lower in the case of WA, demonstrating an anti-metastatic efficacy at a dose as low as 0.1 mg/kg body weight (Thaiparambil et al., 2011). At 4 mg WA/kg body weight, there was a reduction in lung metastatic nodules of more than 70% (Thaiparambil et al., 2011). The same researchers showed in a different trial that oral treatment of ethanol extract of *Withania somnifera* standardized for WA (1, 4, and 8 mg/kg body weight, three times a week for four weeks) inhibited lung metastases caused by breast fat pad injection of 4T1 (Yang et al., 2013). In order to ascertain if this little molecule is in charge of *Withania somnifera*'s anti-metastatic action, mice were also given intraperitoneal treatments three times a week at doses of 1, 4, and 8 mg/kg of WA (Yang et al., 2013). WA and *Withania somnifera*'s ethanol extract both reduced the multiplicity of lung metastases(Yang et al., 2013). Only at the 8 mg/kg dose did the *Withania somnifera* ethanol extract show a marginally greater effectiveness than WA (Yang et al., 2013). Additionally, spontaneous lung metastases occur in the MMTV-neu animals. When 0.1 mg WA/mouse was administered intraperitoneally three times a week, the incidence of pulmonary metastasis was reduced by approximately 73% (Hahm et al., 2013). All of these investigations showed that WA has the ability to prevent lung metastases. The main reason for morbidity and death in breast cancer patients is metastasis, or the spread of the disease to other organs. In comparison to other sites (8–47%), the skeleton is the most frequently colonized location in each breast cancer subtype (ranging from 43 to 71%), while the brain, liver, lungs, and bones may also be affected (Hahm et al., 2013). It would be valuable to investigate whether WA has anti-metastatic properties beyond pulmonary metastases in the future.

Treatment with WA affects cellular processes that are relevant to cancer (cancer hallmarks)

Self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, infinite replicative potential, prolonged angiogenesis, and evasion of apoptosis were the initial six hallmarks of cancer (Stan et al., 2008). New characteristics such as avoiding immune destruction, tumor promoting inflammation, deregulating cellular energetics, and genome instability and mutation have now been added to this list (Hanahan & Weinberg, 2011). Research has shown that WA has an inhibitory effect on a number of cancer hallmarks in breast cancer, are briefly covered here.

(A) WA therapy inhibits the growth of breast cancer cells and stops their cycle.

WA inhibits cell viability in cellular models of breast cancer utilizing MCF-7 and MDA-MB-231 cells with an IC50 ranging from 1.5 to 2.0 μmol/L after a 24-hour treatment period (Stan et al., 2008). In addition, WA strongly decreased the proliferation of MCF-7 cells driven by estrogen. The anti-cancer action of WA was linked to the inhibition of PCNA or Ki-67 expression in the MDA-MB-231 xenograft model, but not in the MMTV-neu or MNU-rat models (Stan et. al., 2008)(Samanta et al., 2017) (Nagalingam et al., 2015) (Hahm et al., 2013). In MCF-7 and MDA-MB-231 cells, WA treatment caused an irreversible G2/M cell cycle arrest. This was accompanied by a reduction in the levels of important cell cycle regulators, such as cyclin-dependent kinase 1 (Cdk1), cell division cycle 25C (Cdc25C), and/or Cdc25B proteins. This accumulation of phosphorylated (inactive) tyrosine 15 Cdk1 (Stan et al., 2008). In MDA-MB-231 cells, overexpression of the Cdc25C protein prevented WA-mediated G2/M phase cell cycle arrest partially but statistically significantly (Stan et al., 2008). Other scientists verified the G2/M arrest from WA therapy (Zhang et al., 2011). In MCF-7, SUM159, and SK-BR-3 cells, another investigation demonstrated mitotic arrest after WA treatment, which was connected to a drop in β-tubulin protein levels (Antony et al., 2014). Withanone and withanolide A, the naturally occurring C6, C7-epoxy analogs of WA, were unable to induce mitotic arrest in these cells (Antony et al., 2014). When compared to breast cancer cells exhibiting cancer cell-specific mitotic arrest by WA, the non-tumorigenic normal mammary epithelial cell line MCF-10A exhibited greater resistance to this drug (Antony et al., 2014). Spindle morphology was similarly disrupted by WA treatment (Antony et al., 2014). The mechanism by which WA causes G2/M arrest appears intricate and might involve other regulators, as peptidyl-prolyl cis/trans isomerase 1 (Pin1) has been shown to do (Samanta et al., 2019). In MCF-7 and SK-BR-3 cells, it was demonstrated that WA downregulates Pin1, and that its ectopic expression decreased G2 and/or mitotic arrest caused by WA (Samanta et al., 2019). Pin1 overexpression boosted WA-induced apoptosis in MCF-7 cells but not in the SK-BR-3 cell line. Moreover, enhanced Ser10 phosphorylation of histone H3 in vivo indicated that WA-mediated chemoprevention of breast cancer in MMTV-*neu* and MNU-rat models was linked to the build-up of mitotic cells (Samanta et al., 2017). All of these results point to the possibility that the G2/M phase cell cycle arrest may play a significant role in the WA's ability to inhibit the proliferation of human breast cancer cells (Samanta et al., 2017) (Hahm et al., 2013) (Stan & Zeng, 2008) (Zhang et al., 2011) (Antony et al., 2014) (Suman et al., 2018).

(B) WA treatment of breast cancer cells inhibits angiogenesis, cell migration and invasion, and the epithelial to mesenchymal transition (EMT).

In tumor metastasis, angiogenesis, cell migration and invasion, and EMT are essential processes (Harris et al., 1995) (Kumar & Golani, 2020) . WA has an inhibitory effect on each of these pro-metastatic pathways, according to published data. Thaiparambil et al. were the first to show that in vitro wound healing and Matrigel invasion experiments may decrease MDA-MB-231 cell invasion (Thaiparambil et al., 2011).

It's interesting to note that WA was able to prevent MDA-MB-231 cell migration at non-cytotoxic, non-apoptotic concentrations as low as 27 nM (Thaiparambil et al., 2011). After treatment with WA, imaging studies showed that vimentin was phosphorylated on Ser56 and that there was a promotion of perinuclear vimentin accumulation followed by fast vimentin depolymerization in breast cancer cells (Thaiparambil et al., 2011). One of the essential proteins in EMT is the vitellin protein (Kumar & Golani 2020). In a different investigation, it was discovered that treating patients with WA reduced the amounts of both the transmembrane and cleaved forms of Notch1, while activating the transcription factors Notch2 and Notch4 (Lee et al., 2013). In MDA-MB-231 and MDA-MB-468 cells, knockdown of both Notch2 and Notch4 increased WA-mediated inhibition of cell migration (Lee et al., 2013). This work revealed an undesirable result in which the inhibitory effect of WA on the migration of breast cancer cells was hindered by the activation of Notch2 and Notch4 (Lee et al., 2013). The inhibitory effect of WA on MDA-MB-231 cell invasion capacity was further demonstrated using a single-cell collagen invasion experiment (Szarc et al., 2014). A decrease in the expression of numerous extracellular matrix-degrading proteases (uPA, PLAT, ADAM8), cell adhesion molecules (integrins, laminins), and several pro-inflammatory mediators (TNFSF12, IL6, ANGPTL2, CSF1R) was found by gene expression profiling in this work (Szarc et al., 2014). The applicability of these WA-induced changes in gene expression to cell lines other than MDA-MB-231 is still uncertain.

The inhibitory impact of WA was demonstrated using an experimental model of EMT when non-tumorigenic MCF-10A cells were treated with tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β) (Lee et al., 2016). Combining TNF-α and TGF-β treatments partially overrode WA treatment's inhibition of experimental EMT and cell migration (Lee et al., 2016). One of the characteristics of the EMT phenotype is the downregulation of E-cadherin, and breast cancer cells treated to WA showed either a transient (MDA-MB-231) or sustained (MCF-7) increase of E-cadherin protein expression (Kumar & Golani, 2020) (Lee et al., 2016). Additionally, as compared to matched controls, the vimentin protein level was significantly lower in the MMTV-neu tumors and MDA-MB-231 xenografts from WA-treated mice (Lee et al., 2016) . Lastly, the anti-angiogenic impact of WA was

assessed using human umbilical vein endothelial cells (Mohan et al., 2004). Nevertheless, in MMTV-neu or MNU-rat models, WA treatment did not significantly change the number of CD31-positive blood vessels, a hallmark of neo-angiogenesis (Samanta et al., 2017) (Hahm et al., 2013).

(C) WA modifies the response to DNA damage in breast cancer cells

The DNA damage response mechanism for maintaining genomic integrity is influenced by ataxia telangiectasia and Rad3-related (ATR)-checkpoint kinase 1 (CHK1) signaling (Awasthi et al., 2015) (Fokas et al., 2014). Replication stress during cell division or a genotoxic insult activates the ATR kinase, which is involved in the S and G2 phases of the cell cycle (Awasthi et al., 2015) (Fokas et al., 2014). When human breast cancer cells (MCF-7, MDA-MB-231, and SUM159) were exposed to WA, both transcriptional and post-transcriptional processes resulted in the decrease of protein levels and the phosphorylation of ATR and CHK1 (Hahm et al., 2020). The WA-mediated G2/M arrest was eliminated by forced expression of CHK1, while histone H3 Ser10 phosphorylation was elevated (Hahm et al., 2020). In the breast tumors of WA-treated MMTV-neu mice, there was a tendency toward a decrease in the protein level of ATR, although the difference was not statistically significant (Hahm et al., 2020). Although our study has identified therapeutically important sensitization of MDA-MB-231 and SUM159 cells to cisplatin-induced growth inhibition, the in vivo effects of this potential combination treatment have not yet been determined (Hahm et al., 2020). To find out if WA therapy impacts additional DNA damage response mechanisms, more research is required.

(D) WA treatment induces apoptosis in breast cancer cells

The pro-apoptotic impact of WA is the most researched (Thaiparambil et al., 2011) (Nagalingam et al., 2015) (Liu et al., 2019) (Zhang et al., 2011) (Zhang et al., 2012) (Eun-Ryeong Hahm et al., 2014) (Royston et al., 2017) (Sehrawat et al., 2020). By using western blotting to measure the release of DNA fragments into the cytoplasm and detect poly (ADP-ribose) polymerase breakage, we were the first to show that WA induced apoptosis in MCF-7 and MDA-MB-231 cells (Cai et al., 2014). The apoptotic induction that coincided with the in vivo growth suppression of the MDA-MB-231 xenograft was demonstrated by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) test (Stan et al., 2008). An increase in TUNEL-positive apoptotic cells was also linked to WA-mediated chemoprevention of breast cancer in MMTV-neu and MNU-rat models (Samanta et al., 2017) (Hahm et al., 2013). Since then, numerous researchers have verified WA's proapoptotic impact in cell lines other than MCF-7 and MDA-MB-231 (Thaiparambil et al., 2011) (Nagalingam A, Kuppusamy P, Singh SV, Sharma D, 2015) 27, (Zhang et al., 2011) (Zhang et al., 2012). Reactive oxygen species (ROS) production is mechanistically linked to WA's proapoptotic action (Liu et al., 2019) (Eun-Ryeong Hahm et al., 2014). At complex III of the mitochondrial electron transport chain, WA-mediated suppression of oxidative phosphorylation (OXPHOS) resulted in the production of ROS (Eun-Ryeong Hahm et al., 2014). According to MDA-MB-231 and MCF-7 cells, but not HMEC, a normal human mammary epithelial cell line, WA only produces ROS in cancer cells (Eun-Ryeong Hahm et al., 2014). Additionally, resistant to WA's pro-apoptotic effects were the HMEC cells (Eun-Ryeong Hahm et al., 2014). Depletion of mitochondrial DNA (Rho-0 cells) or overexpression of Cu, Zn-superoxide dismutase (SOD1) in MDA-MB-231 or MCF-7 cells similarly resulted in resistance to WA-induced ROS generation, collapse of mitochondrial membrane potential, and death (Eun-Ryeong Hahm et al., 2014). It has also been proposed that one of the key roles for ROS-dependent activation of Bak and/or Bax in WA-induced apoptosis (Eun-Ryeong Hahm et al., 2014). Though we have observed a decrease in complex III assembly in MCF-7 and SUM159 cells but not in MDA-MB-231 following treatment with WA, as assessed by native blue gel electrophoresis, the exact mechanism by which WA suppresses complex III activity is still unclear (Sehrawat et al., 2020). Additionally, chemically induced mitochondrial fusion in breast cancer cells was prevented by WA therapy, which was linked to the downregulation of proteins (full length optic atrophy protein 1; OPA1, mitofusin1, and mitofusin2). Exposure to WA reduced the amount of the mitochondrial fission-regulating protein dynamin-related protein 1 (DRP1), and its lack as well as OPA1 knockdown lessened the apoptotic effect of WA (Sehrawat et al., 2020). According to one study, treating WA with WA may cause paraptosis, a type of cell death that differs morphologically from apoptosis or autophagy (Ghosh et al., 2016). However, it is unknown what mechanism causes this reaction or whether it has any practical significance in vivo.

(E) WA therapy inhibits aerobic glycolysis in breast cancer cells

In year 2011 observed the addition of metabolic reprogramming, which includes increased glycolysis in tumor cells, to the list of cancer hallmarks, despite the fact that Dr. Otto Warburg first described this phenomenon in the 1950s (Hanahan & Weinberg, 2011) (Maurer, 2012). While normal cells obtain their ATP from OXPHOS, cancer cells develop an addiction to aerobic glycolysis in order to meet their energy demands (Maurer, 2012) (Dias et al., 2019). Using unbiassed global metabolomics, we were able to report a decrease in the levels of several glycolysis and tricarboxylic acid cycle intermediates in the plasma and/or mammary tumor tissue of WA treated MMTV-neu mice, in comparison to control mice (Hahm et al., 2013). Numerous enzyme proteins associated with the tricarboxylic acid cycle and glycolysis were also less expressed in the tumors of WA-treated MMTV-neu animals than in control mice. The MMTV-neu model is not the only one to exhibit WA-mediated regulation of glycolysis; the MNU-rat model also showed a reduction in plasma lactate levels (Samanta et al., 2017) (Hahm et al., 2013). Moreover, the administration of WA led to acetyl-CoA and glutamine levels being suppressed (Samanta et al., 2017) (Hahm et al., 2013). The foundation of fatty acid production is acetyl CoA. In HER2-enriched or triple-negative breast tumors, there has been evidence of increased fatty acid production or increased cholesterol uptake. Therefore, it is plausible that treatment with WA prevents breast cancer cells from synthesizing fatty acids; however, further investigation is required to verify this theory.

Furthermore, it is still unclear what the molecular underpinnings of WA's metabolic inhibition are.

Interestingly, WA treatment prevents MCF-7 and MDA-MB-231 cells from undergoing OXPHOS (Eun-Ryeong Hahm et al., 2014). Basal oxygen consumption rate (OCR), a gauge of OXPHOS, was found by seahorse flux analysis to be comparatively greater in the MCF-7 cell line than in MDA-MB-231 (Eun-Ryeong Hahm et al., 2014). However, after being exposed to 2.5 and 5 μmol/L WA for 4 hours, both cells showed a statistically significant drop in baseline OCR (Eun-Ryeong Hahm et al., 2014). The MDA-MB-231 cell line exhibited a comparatively stronger inhibitory effect of WA on basal OXPHOS in contrast to MCF-7 (Eun-Ryeong Hahm et al., 2014). In MDA-MB-231 and MCF-7 cells, WA administration led to a statistically significant decrease in reserve OXPHOS, particularly at the 5 μmol/L dose (Eun-Ryeong Hahm et al., 2014). It is unknown, nevertheless, if administering WA therapy suppresses OXPHOS in HER2-enriched breast cancer cell lines or in vivo in breast cancer cells of various subtypes. Furthermore, we are aware that WA interferes with electron flow in complex III of the electron transport chain, which could account for the phytochemical's ability to produce ROS.

(F) WA's immune-modulating action

Through their suppression of T cell function and interaction with tumor-associated macrophages, myeloid-derived suppressor cells (MDSC) are crucial in the development of tumors (Nagaraj & Gabrilovich, 2010). The outcome of MDSC's interaction with tumor-associated macrophages to enhance IL-10 production is one of their strategies for evading tumor immunity (Dias et al., 2019). It has been established that WA therapy inhibits the production of IL-10 and MDSC (Sinha & Ostrand, 2013). Macrophages release TNFα and IL-6, which promote MDSC accumulation and function (Nagaraj & Gabrilovich, 2010). Treatment with WA also prevented macrophages from secreting TNFα and IL-6 (Sinha & Ostrand, 2013). Granulocytic MDSC were suppressed when 4T1 mouse mammary tumor-bearing mice were given oral WA (1–8 mg/kg body weight, three times/week) (Sinha & Ostrand, 2013). In a different investigation, treating CD8+ and CD4+ T cells with WA reduced their production of IL-2, IL-4, IL-6, and IFN-γ in response to mitogens (Gambhir et al., 2015). There have also been reports of *Withania somnifera* extract's immune-modulatory properties, which may be related to WA (Malik et al., 2009) (L, Davis G, 2002). It is yet unknown, though, how WA would affect the in vivo immune modulatory processes in preclinical models of various breast cancer subtypes.

Targets of WA molecules in breast cancer cells

In order to trigger its anticancer effects, such as inducing apoptosis and inhibiting cell proliferation, migration/invasion, and bCSC self-renewal, WA targets a variety of transcription factors, receptors, and kinases.

(A) FOXO3a

The forkhead box transcription factor FOXO3a was the first molecular target of WA in breast cancer cells to be discovered (Stan et al., 2008). FOXO3a has been extensively linked to several solid tumors, such as breast cancer, and current data suggests that this transcription factor has a tumor-suppressive role. In MCF-7 cells, FOXO3a knockdown produced a significant but incomplete defense against WA-mediated apoptosis that involved its downstream pro-apoptotic target, Bim (Stan et al., 2008).

(B) STAT3

Breast cancer development, proliferation, metastasis, and chemoresistance have all been linked to STAT3 overexpression and constitutive activation (Ma et al., 2020). In breast cancer cells, WA treatment also inhibits the activity of STAT3, another transcription factor (Lee et al., 2010). In MCF-7 and MDA-MB-231 cells, WA administration has been demonstrated to prevent the constitutive and/or IL-6-induced activation of STAT3, as well as the phosphorylation of its upstream regulator, Janus-activated kinase 2 (Lee et al., 2010). When WA was applied to MDA-MB-231 or MCF-7 cells, it also suppressed (a) STAT3 transcriptional activity, whether or not IL-6 stimulation was present; (b) STAT3 dimerization, at least in MDA-MB-231 cells; and (c) phosphorylated STAT3 nuclear translocation in both cells (Lee et al., 2010). WA-inhibited cell invasion was partially prevented in this instance by the IL-6-mediated activation of STAT3.

(C) ERα

The pro-tumorigenic activity of ERα in breast cancer has been extensively researched (Lamb et al., 2019). The effects of WA treatment on MCF-7 cells' growth inhibition and induction of apoptosis were considerably reduced by the ERα ligand 17β-estradiol (E2) (Eun-Ryeong Hahm et al., 2011). E2 significantly reduced the effect of WA on MCF-7 cells, which showed reduced protein levels of ERα (but not ERβ) and the ERα controlled gene product pS2 (Eun-Ryeong Hahm et al., 2011). In the MDA- MB-231 cell line, overexpression of ERα prevented WA-mediated apoptosis somewhat but statistically significantly, but not the G2/M phase cell cycle arrest (Eun-Ryeong Hahm et al., 2011). Another group of researchers validated the downregulation of ERα protein expression in MCF-7 cells after treatment with WA (Zhang et al., 2011).

(E) p53

Tumor suppressor p53 is well known for its ability to control cell cycle and apoptosis in response to various stimuli (Slee et al., 2004). In MCF-7 cells, WA treatment resulted in both induction and enhanced Ser15 phosphorylation of p53 (activation); nevertheless, RNA interference of this tumor suppressor, at least in this cell line, only slightly protected against WA-induced apoptosis (Eun-Ryeong Hahm et al., 2011).

(F) Receptors and transcription factors linked to bCSC maintenance

The stemness-related genes Oct4, SOX-2, and Nanog showed downregulated expression at the 72-hour mark in SUM159 cells, whereas only SOX-2 mRNA was suppressed following a 24-hour WA treatment in MCF-7 cells, according to expression profiling (Kim and Singh, 2015). Nevertheless, it is still unknown how exactly these stemness-related genes contribute to WA-mediated suppression of bCSC.

In MCF-7 cells, stemness is solely induced by overexpressing the urokinase-type plasminogen activator receptor (uPAR) (jo et al., 2010). Partial but significant protection against bCSC inhibition by WA was provided by

overexpressing uPAR. It's interesting to note that Krüppel-like factor 4 (KLF4) was induced by WA therapy. Expression in MCF-7 and SUM159 cells, which has been demonstrated to be necessary for the maintenance of bCSC and breast cancer cell motility and invasion (Yu et al., 2011). Its knockdown by KLF4-targeted siRNA transfection enhanced bCSC suppression by WA (Kim and Singh, 2015). Triple negative and hormone receptor positive breast tumors are linked to the Hedgehog pathway (Bhateja et al., 2019). WA was found to be an inhibitor of the Hedgehog pathway in one investigation, however no tests were done to ascertain the functional significance of this discovery (Yoneyama et al., 2015).

(G) Kinases

Studies on the impact of WA have been conducted using breast cancer cells to examine extracellular signal-regulated kinases (ERK), p38 mitogen-activated kinase, and c-Jun N-terminal kinases (JNK). In both cells, treatment with WA activated all three kinases (Eun-Ryeong Hahm et al., 2015). Manganese-superoxide dismutase overexpression provided some protection against WA-mediated ERK hyperphosphorylation (activation), but not against JNK or p38 MAPK (Eun-Ryeong Hahm et al., 2015).

ERK and p38 MAPK pharmacological inhibition greatly increased the amount of apoptosis induced by WA treatment in MCF-7 cells (Eun-Ryeong Hahm et al., 2015). On the other hand, JNK inhibition somewhat reduced WA-mediated apoptosis in MCF-7 cells (Eun-Ryeong Hahm et al., 2015). There was no significant effect of ERK or JNK inhibition on WA-induced apoptosis in the SUM159 cell line. It is unknown, therefore, how WA affects these pathways in normal mammary cells or HER2-enriched breast cancer cells. In a different investigation, downregulation of ERBB2 was demonstrated using a relatively high dosage of WA (10 μmol/L) (Liu et al., 2016). Breast cancer cells were become more sensitive to WA by overexpression of ERBB2 (Liu et al., 2016). One of the downstream effects of WA therapy was the inhibition of AKT (Liu et al., 2016). Following treatment with WA, there was also a report of increased phosphorylation of ribosomal S6 kinase (RSK) in breast cancer cells (Nagalingam et al., 2015). When WA activated RSK, ETS-like transcription factor 1 and C-EBP homologous protein kinase pathways were triggered. This increased expression of death receptor 5 resulted in apoptotic cell death (Nagalingam et al., 2015).

(H) Members of the Inhibitor of Apoptosis (IAP) family

Members of the IAP family of proteins block caspases to control apoptosis. In MCF-7 and MDA-MB-231 cells, treatment with WA led to the downregulation of XIAP, cIAP-2, and survivin protein levels (Eun-Ryeong Hahm et al., 2014). In both cell lines, overexpression of XIAP, survivin, and cIAP-2 reduced the induction of apoptosis induced by WA (Eun-Ryeong Hahm et al., 2014). It's interesting to note that WA treatment inhibited the growth of MDA-MB-231 xenografts and was linked to a statistically significant downregulation of only survivin protein expression (Eun-Ryeong Hahm et al., 2014). It is unknown what part the IAP family members play in vivo in WA's protection of breast cancer.

(I) Proteins that regulate autophagy

An evolutionary conserved mechanism for recycling cellular macromolecules and organelles like mitochondria is autophagy (Romero et al., 2019). Members of the autophagy-related gene (ATG) family (Romero et al., 2019). strictly regulate this process. The induction of autophagy in MCF-7 and MDA-MB-231 cells after treatment with WA was originally shown in our lab (Hahm et al., 2013). Nevertheless, this mechanism is not exclusive to cancer, since WA was also found to induce autophagy in the MCF-10A cell line (Hahm et al., 2013). WA treatment-induced inhibition of MDA-MB-231 and MCF-7 cell survival was unaffected by either genetic repression of ATG5 or pharmacological suppression with 3-methyl adenine (Hahm et al., 2013). Other researchers later demonstrated that WA induced autophagy in breast cancer cells (Liu et al., 2019) (Muniraj et al., 2019).

(J) Proteins modified covalently

Upon being an electrophile (Michael acceptor), WA can react directly with one or more cysteine residues found in many proteins. Using endothelial cells, Bargagna-Mohan et al. (Bargagna et al., 2010) were the first to show how WA treatment (5 μmol/L) modified the cysteine-328 of the vimentin protein. After treating MCF-7 cells with 2 μmol/L WA, further work from our own laboratory demonstrated covalent alteration of cysteine-303 of β-tubulin (Antony et al., 2014). In this work, we employed NMR to show that the A-ring enone in WA was extremely reactive to the nucleophile cysteamine and quickly gave way to irreversible nucleophilic addition (Antony et al., 2014). Based on molecular docking, the WA-binding pocket was identified as being on the β-tubulin surface, with three distinct features: a hydrophobic floor, a hydrophobic wall, and a charge-balanced hydrophilic entry. These findings provide fresh perspectives on the process by which WA causes growth arrest in breast cancer cells (Antony et al., 2014). Another mechanism of NF-κB suppression described was covalent alteration of cysteine-179 of IKKβ following treatment of human embryonic kidney HEK293T cells with 5 μmol/L WA (Heyninck et al., 2014). Gambhir et al. reported covalent alteration of the cysteine-62 residue using a synthetic NF-κB-p50 peptide (Gambhir et al., 2015). In a recent study, Grossman et al. (Grossman et al., 2017) mapped the proteome-wide cysteine modification by WA using chemo-proteomic platforms. However, other previously known modifications (e.g., vimentin, β-tubulin or IKKβ) could not be validated in the study by Grossman et al (Grossman et al., 2017), 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 (Suman et al., 2018).

II. Conclusions, Directions for the Future, and Information Gaps

The ideal attributes for a clinically developed chemopreventive intervention are safety, efficacy, target tissue bioavailability, oral bioavailability, and selectivity towards cancer cells. Based on the preclinical research covered in this article, WA satisfies each of these requirements. Before WA is

investigated clinically, there are still important knowledge gaps that must be closed. Initially, it is necessary to ascertain the kinetics of WA clearance and bioavailability in mammary and tumor tissues in order to optimize the dosing schedule. Although xenograft studies have demonstrated an in vivo therapeutic response of WA against basal-like human breast cancer cells, cellular in vitro studies indicate that basal-like breast cancer cells are sensitive to WA. Nevertheless, it is crucial to investigate whether development of this subtype of breast cancer is prevented by WA administration. Studies on cellular in vitro mechanistics have predominantly examined MCF-7 luminal type and basal-like MDA-MB-231 cells. To increase our understanding of the mechanisms involved, more research is required using HER2-enriched breast cancer cell lines, such as SK-BR-3.

While WA has been shown to reduce cell viability and cause mitotic arrest in basal, luminal, and Her2-enriched breast cancer cells (Antony et al., 2014). However, the preclinical data are strong enough to move WA toward a phase 1 study. One present drawback is that the US Food and Drug Administration has not yet given WA approval.

REFERENCES

[1] Al-hajj, M., Wicha, M. S., Benito-hernandez, A., Morrison, S. J., & Clarke, M. F. (2003). Prospective identification of tumorigenic breast cancer cells. https://doi.org/10.1073/pnas.0530291100

[2] Antony, M. L., Lee, J., Hahm, E. R., Kim, S. H., Marcus, A. I., Kumari, V., Ji, X., Yang, Z., Vowell, C. L., Wipf, P., Uechi, G. T., Yates, N. A., Romero, G., Sarkar, S. N., & Singh, S. V. (2014). Growth arrest by the antitumor steroidal lactone withaferin a in human breast cancer cells is associated with down-regulation and covalent binding at cysteine 303 of β-tubulin. Journal of Biological Chemistry, 289(3), 1852–1865. https://doi.org/10.1074/jbc.M113.496844

[3] Awasthi, P., Foiani, M., & Kumar, A. (2015). CELL SCIENCE AT A GLANCE ATM and ATR signaling at a glance. 4255–4262. https://doi.org/10.1242/jcs.169730

[4] Bargagna-Mohan P, Paranthan RR, Hamza A, Dimova N, Trucchi B, Srinivasan C, Elliott GI, Zhan CG, Lau DL, Zhu H, Kasahara K, Inagaki M, Cambi F, Mohan R. Withaferin A targets intermediate filaments glial fibrillary acidic protein and vimentin in a model of retinal gliosis. J Biol Chem. 2010 Mar 5;285(10):7657-69. doi: 10.1074/jbc.M109.093765. Epub 2010 Jan 4. PMID: 20048155; PMCID: PMC2844212.

[5] Brien, C. S. O., Farnie, G., Howell, S. J., & Clarke, R. B. (2011). Breast Cancer Stem Cells and Their Role in Resistance to Endocrine Therapy. 91–103. https://doi.org/10.1007/s12672-011-0066-6

[6] Cai, Y., Sheng, Z. Y., Chen, Y., & Bai, C. (2014). Effect of withaferin A on A549 cellular proliferation and apoptosis in non-small cell lung cancer. Asian Pacific Journal of Cancer Prevention, 15(4), 1711–1714. https://doi.org/10.7314/APJCP.2014.15.4.1711

[7] Cauley JA, Norton L, Lippman ME, Eckert S, Krueger KA, Purdie DW, et al. (2001). Continued breast cancer risk reduction in postmenopausal women treated with raloxifene: 4-year results from the MORE trial: Multiple outcomes of raloxifene evaluation. Breast Cancer Res Treat 2001, 65(2), 125– 34. https://doi.org/10.1023/a:1006478317173

[8] Chan MM, Lu X, Merchant FM, Iglehart JD, Miron PL. Gene expression profiling of NMU-induced rat mammary tumors: cross species comparison with human breast cancer. Carcinogenesis. 2005 Aug;26(8):1343-53. doi: 10.1093/carcin/bgi100. Epub 2005 Apr 21. PMID: 15845649.

[9] Choudhary, D., Bhattacharyya, S., & Bose, S. (2017). Efficacy and Safety of Ashwagandha (Withania somnifera (L.) Dunal) Root Extract in Improving Memory and Cognitive Functions. Journal of Dietary Supplements, 14(6), 599–612. https://doi.org/10.1080/19390211.2017.1284970

[10] Committee, I. E., Strelaxin, C., Pvt, P. H., & Adr, N. (2013). Effects of Withania somnifera in patients of schizophrenia: A randomized , double blind , placebo controlled pilot trial study. 45(4), 417–418. https://doi.org/10.4103/0253-7613.115012

[11] Dai T, Jiang W, Guo Z, Wang Z, Huang M, Zhong G, Liang C, Pei X, Dai R. Studies on oral bioavailability and first-pass metabolism of withaferin A in rats using LC-MS/MS and Q-TRAP. Biomed Chromatogr. 2019 Sep;33(9):e4573. doi: 10.1002/bmc.4573. Epub 2019 May 26. PMID: 31062367.

[12] Dias, A. S., Almeida, C. R., Helguero, L. A., & Duarte, I. F. (2019). ScienceDirect Metabolic crosstalk in the breast cancer microenvironment. European Journal of Cancer, 121, 154–171. https://doi.org/10.1016/j.ejca.2019.09.002

[13] Eun-Ryeong Hahm and Shivendra V. Singh et al. (2014). Withaferin A-induced apoptosis in human breast cancer cells is associated with suppression of inhibitor of apoptosis family protein expression. NIH Public Access, 334(1), 101–108. https://doi.org/10.1016/j.canlet.2012.08.026.Withaferin

[14] Eun-Ryeong Hahm, Joomin Lee, and S. V. S. (2015). Role of Mitogen-Activated Protein Kinases and Mcl-1 in Apoptosis Induction by Withaferin A in Human Breast Cancer Cells. NIH Public Access, 53(11), 907–916. https://doi.org/10.1002/mc.22050.Role

[15] Eun-Ryeong Hahm, Joomin Lee, Yi Huang, and S. V. S. (2011). Withaferin A Suppresses Estrogen Receptor-α Expression in Human Breast Cancer Cells. 50(8), 614–624. https://doi.org/10.1002/mc.20760.Withaferin

[16] Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, Vogel V, Robidoux A, Dimitrov N, Atkins J, Daly M, Wieand S, Tan-Chiu E, Ford L, Wolmark N. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst. 1998 Sep 16;90(18):1371-88. doi: 10.1093/jnci/90.18.1371. PMID: 9747868.

[17] Fokas, E., Prevo, R., Hammond, E. M., Brunner, T. B., Mckenna, W. G., & Muschel, R. J. (2014). Targeting ATR in DNA damage response and cancer therapeutics. Cancer Treatment Reviews, 40(1), 109–117. https://doi.org/10.1016/j.ctrv.2013.03.002

[18] Gambhir, L., Checker, R., Sharma, D., Thoh, M., Patil, A., Degani, M., Gota, V., & Sandur, S. K. (2015). Thiol dependent NF-κB suppression and inhibition of T-cell mediated adaptive immune responses by a naturally occuring steroidal lactone withaferin a. Toxicology and Applied Pharmacology. https://doi.org/10.1016/j.taap.2015.09.014

[19] Ghosh, K., De, S., Das, S., Mukherjee, S., & Sengupta, S. (2016). Withaferin A Induces ROS-Mediated Paraptosis in Human Breast Cancer Cell-Lines. 1–15. https://doi.org/10.1371/journal.pone.0168488

[20] Goss, P. E., Ingle, J. N., Cheung, A. M., Chlebowski, R. T., Wactawski-wende, J., Mctiernan, A., Robbins, J., Johnson, K. C., Martin, L. W., Winquist, E., Sarto, G. E., Garber, J. E., Fabian, C. J., Pujol, P., Maunsell, E., Farmer, P., Gelmon, K. A., Tu, D., & Richardson, H. (2011). new england journal. 2381–2391.

[21] Gottardis, M. M., Jordan, V. C., Gottardis, M. M., & Jordan, V. C. (1987). Antitumor Actions of Keoxifene and Tamoxifen in the N - Nitrosomethylurea-induced Rat Mammary Carcinoma Model Antitumor Actions of Keoxifene and Tamoxifen in the TV-Nitrosomethylurea- induced Rat Mammary Carcinoma Model1 Treatment of Rats. 4020–4024.

[22] Grossman EA, Ward CC, Spradlin JN, Bateman LA, Huffman TR, Miyamoto DK, et al. Covalent ligand discovery against druggable hotspots targeted by anti-cancer natural products. Cell Chem Biol 2017;24:1368–76. [PubMed: 28919038]

[23] Hahm, E., Kim, S., Singh, K. B., Singh, K., & Shivendra, V. (2021). Withaferin A inhibits in vivo growth of breast cancer cells accelerated by Notch2 knockdown. HHS Public Access, 13(9), 721–734. https://doi.org/10.1158/1940-6207.CAPR-20-0259.A

[24] Hahm, E., Lee, J., Abella, T., Singh, S. V, & Korea, S. (2020). Withaferin A inhibits expression of ataxia telangiectasia and Rad3-related kinase and enhances sensitivity of human breast cancer cells to cisplatin. 58(11), 2139–2148. https://doi.org/10.1002/mc.23104.Withaferin

[25] Hahm, E., Lee, J., Kim, S., Sehrawat, A., Arlotti, J. A., Shiva, S. S., Bhargava, R., & Singh, S. V. (2013). Metabolic Alterations in Mammary Cancer Prevention by Withaferin A in a Clinically Relevant Mouse Model. https://doi.org/10.1093/jnci/djt153

[26] Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. Cell, 144(5), 646–674. https://doi.org/10.1016/j.cell.2011.02.013

[27] Harris, A. L., Fox, S., Leek, R., Zhang, H., Scott, P., Bicknell, R., & Gatter, K. (1995). Breast cancer angiogenesis: therapy target and prognostic factor. European Journal of Cancer, 31(5), 831-832.

[28] Heyninck K, Lahtela-Kakkonen M, Van der Veken P, Haegeman G, Vanden Berghe W. Withaferin A inhibits NF-κB activation by targeting cysteine 179 in IKKβ. Biochem Pharmacol 2014;91:501– 9. [PubMed: 25159986]

[29] Joomin Lee, Eun-Ryeong Hahm, Adam Marcus, and S. V. S. (2016). Withaferin A Inhibits Experimental Epithelial-Mesenchymal Transition in MCF-10A Cells and Suppresses Vimentin Protein Level in Vivo in Breast Tumors Joomin. 54(6), 417–429. https://doi.org/10.1002/mc.22110.Withaferin

[30] Kim SH and Singh SV (2015). Mammary cancer chemoprevention by withaferin A is accompanied by in vivo suppression of self-renewal of cancer stem cells. NIH Public Access, 7(7), 738–747. https://doi.org/10.1158/1940-6207.CAPR-13-0445.Mammary

[31] Kumar A, Golani A, K. L. (2020). EMT in breast cancer metastasis: an interplay of microRNAs, signaling pathways and circulating tumor cells. 1, 979–1010.

[32] L, Davis G, K. (2002). Effect of Withania somnifera on cell mediated immune responses in mice. Ournal of Experimental & Clinical Cancer Research, 21(4), 585–590.

[33] Lamb, C. A., Vanzulli, S., & Lanari, C. L. M. (2019). Hormone receptors in breast cancer: more than estrogen receptors.

[34] Lee J, Sehrawat A, and Singh sv. (2013). Withaferin A causes activation of Notch2 and Notch4 in human breast cancer cells. NIH Public Acces, 136(1), 45–56. https://doi.org/10.1007/s10549-012-2239-6.

[35] Lee, J., Hahm, E. R., & Singh, S. V. (2010). Withaferin A inhibits activation of signal transducer and activator of transcription 3 in human breast cancer cells. Carcinogenesis, 31(11), 1991-1998.

[36] Liu X , Li Y, Ma Q, Wang Y, Song AL. (2019). Withaferin-A Inhibits Growth of Drug-Resistant Breast Carcinoma by Inducing Apoptosis and Autophagy, Endogenous Reactive Oxygen Species (ROS) Production, and Inhibition of Cell Migration and Nuclear Factor kappa B (Nf-κB)/Mammalian Target of Rapamycin (m-. 12(25), 6855–6863. https://doi.org/10.12659/MSM.916931

[37] Liu, W., Barnette, A. R., Andreansky, S., & Landgraf, R. (2016). ERBB2 Overexpression Establishes ERBB3- Dependent Hypersensitivity of Breast Cancer Cells to Withaferin A. 15(November), 2750–2757. https://doi.org/10.1158/1535-7163.MCT-15-0932

[38] Lubet RA, Steele VE, DeCoster R, Bowden C, You M, Juliana MM, Eto I, Kelloff GJ, Grubbs CJ. Chemopreventive effects of the aromatase inhibitor vorozole (R 83842) in the methylnitrosourea-induced mammary cancer model. Carcinogenesis. 1998 Aug;19(8):1345-51. doi: 10.1093/carcin/19.8.1345. PMID: 9744527.

[39] Lubet RA, Steele VE, DeCoster R, Bowden C, You M, Juliana MM, Eto I, Kelloff GJ, Grubbs CJ. Chemopreventive effects of the aromatase inhibitor vorozole (R 83842) in the methylnitrosourea-induced mammary cancer model. Carcinogenesis. 1998 Aug;19(8):1345-51. doi: 10.1093/carcin/19.8.1345. PMID: 9744527.

[40] Ma JH, Qin L, Li X. Role of STAT3 signaling pathway in breast cancer. Cell Commun Signal. 2020 Feb 28;18(1):33. doi: 10.1186/s12964-020-0527-z. PMID: 32111215; PMCID: PMC7048131.

[41] Malik, F., Kumar, A., Bhushan, S., Mondhe, D. M., Pal, H. C., Sharma, R., Khajuria, A., Singh, S., Singh, G., Saxena, A. K., Suri, K. A., Qazi, G. N., & Singh, J. (2009). Immune modulation and apoptosis induction: Two sides of antitumoural activity of a standardised herbal formulation of Withania somnifera. European Journal of Cancer, 45(8), 1494–1509. https://doi.org/10.1016/j.ejca.2009.01.034

[42] Manuscript, A., & Apoptosis, B. (2009). NIH Public Access. 68(18), 7661–7669. https://doi.org/10.1158/0008-5472.CAN-08-1510.Withaferin

[43] Marco A. Velasco-Velázquez, Homsi N, De La Fuente M, Pestell RG.et al. (2012). Breast cancer stem cells. 44(4), 573–577. https://doi.org/10.1016/j.biocel.2011.12.020.Breast

[44] Maurer, P. H. (2012). Warburg O On respiratory impairment in cancer cells. Science 1956, 124(269), 70.

[45] Mohan, R., Hammers, H., Bargagna-Mohan, P., Zhan, X., Herbstritt, C., Ruiz, A., Zhang, L., Hanson, A., Conner, B., Rougas, J., & Pribluda, V. (2004). Withaferin A is a potent inhibitor of angiogenesis. Angiogenesis, 7(2), 115–122. https://doi.org/10.1007/s10456-004-1026-3

[46] Muniraj N, Siddharth S, Nagalingam A, Walker A, Woo J, Győrffy B, Gabrielson E, Saxena NK, Sharma D. Withaferin A inhibits lysosomal activity to block autophagic flux and induces apoptosis via energetic impairment in breast cancer cells. Carcinogenesis. 2019 Sep 18;40(9):1110-1120. doi: 10.1093/carcin/bgz015. PMID: 30698683; PMCID: PMC10893887.

[47] Nagalingam A, Kuppusamy P, Singh SV, Sharma D, S. N. (2015). Mechanistic elucidation of the antitumor properties of withaferin a in breast cancer. 74(9), 2617–2629. https://doi.org/10.1158/0008-5472.CAN-13-2081.Mechanistic

[48] Nagaraj, S., & Gabrilovich, D. I. (2010). Myeloid-Derived Suppressor Cells in Human Cancer THE PHENOTYPE OF MDSC IN MICE AND CANCER. 16(4), 348–353.

[49] Nasimi, R., Azgomi, D., Zomorrodi, A., Nazemyieh, H., Mohammad, S., Fazljou, B., Bazargani, H. S., Nejatbakhsh, F., Jazani, A. M., & Asrbadr, Y. A. (2018). Effects of Withania somnifera on Reproductive System: A Systematic Review of the Available Evidence. 2018. https://doi.org/10.1155/2018/4076430

[50] Palliyaguru, D. L., Singh, S. V, & Kensler, T. W. (2017). HHS Public Access. 60(6), 1342–1353. https://doi.org/10.1002/mnfr.201500756.Withania

[51] Patil D, Gautam M, Mishra S, Karupothula S, Gairola S, Jadhav S, et al. (2013). Determination of withaferin A and withanolide A in mice plasma using high-performance liquid chromatography_tandem mass spectrometry: application to pharmacokinetics after oral administration of Withania somnifera aqueous extract. 2013;80:203–12. J Pharm Biomed Anal, 80, 203–212. https://doi.org/https://doi.org/10.1016/j.jpba.2013.03.001

[52] Pires, N., Gota, V., Gulia, A., Hingorani, L., Agarwal, M., & Puri, A. (2020). Journal of Ayurveda and Integrative Medicine Safety and pharmacokinetics of Withaferin-A in advanced stage high grade osteosarcoma: A phase I trial. Journal of Ayurveda and Integrative Medicine, 11(1), 68–72. https://doi.org/10.1016/j.jaim.2018.12.008

[53] Prospective, A., Double-blind, R., Chandrasekhar, K., Kapoor, J., & Anishetty, S. (2012). Original Article Controlled Study of Safety and Efficacy of a High- Concentration Full-Spectrum Extract of Ashwagandha Root in Reducing Stress and Anxiety in Adults. 34(14), 255–262. https://doi.org/10.4103/0253-7176.106022

[54] Romero MA, Bayraktar Ekmekcigil O, Bagca BG, Avci CB, Sabitaliyevich UY, Zhenisovna TG, et al. Role of autophagy in breast cancer development and progression: Opposite sides of the same coin. Adv Exp Med Biol 2019;1152:65–73. [PubMed: 31456180]

[55] Royston KJ, Udayakumar N, Lewis K, Tollefsbol TO. A Novel Combination of Withaferin A and Sulforaphane Inhibits Epigenetic Machinery, Cellular Viability and Induces Apoptosis of Breast Cancer Cells. Int J Mol Sci. 2017 May 19;18(5):1092. doi: 10.3390/ijms18051092. PMID: 28534825; PMCID: PMC5455001.

[56] Russo, J., Gusterson, B. A., Rogers, A. E., Russo, I. H., Wellings, S. R., & Zwieten, M. J. V. A. N. (1990). Biology of Disease Comparative Study of Human and Rat Mammary. 19(121), 217–218.

[57] Saggam, A., Tillu, G., Dixit, S., Chavan-gautam, P., Borse, S., Joshi, K., & Patwardhan, B. (2020). Withania somnifera (L .) Dunal : A potential therapeutic adjuvant in cancer. 255(February).

[58] Samanta, S. K., Lee, J., Hahm, E., Singh, S. V, Division, S., & Korea, S. (2019). HHS Public Access. 57(7), 936–946. https://doi.org/10.1002/mc.22814.Peptidyl-prolyl

[59] Samanta, S. K., Sehrawat, A., Kim, S., Hahm, E., Shuai, Y., Roy, R., Pore, S. K., Singh, K. B., Christner, S. M., Beumer, J. H., Davidson, N. E., & Singh, S. V. (2017). Disease Subtype – Independent Biomarkers of Breast Cancer Chemoprevention by the Ayurvedic Medicine Phytochemical Withaferin A. 109, 1–13. https://doi.org/10.1093/jnci/djw293

[60] Sarris, J. (2018). Herbal medicines in the treatment of psychiatric disorders : 10 - year updated review. January, 1–16. https://doi.org/10.1002/ptr.6055

[61] Sehrawat, A., Samanta, S. K., Hahm, E., Watkins, S., & Singh, S. V. (2020). Withaferin A-mediated apoptosis in breast cancer cells is associated with alterations in mitochondrial dynamics. HHS Public

Access, 47, 282–293. https://doi.org/10.1016/j.mito.2019.01.003.Withaferin

[62] Shohat B, Gitter S, Abraham A, L. D. (n.d.). Antitumor activity of withaferin A (NSC-101088). Cancer Chemother Rep 1967;51:271–6.

[63] Siegel, R. L., & Miller, K. D. (2020). Cancer Statistics, 2020. 70(1), 7–30. https://doi.org/10.3322/caac.21590

[64] Sinha, P., & Ostrand, S. (2013). Myeloid - derived suppressor cell function is reduced by Withaferin A, a potent and abundant component of Withania somnifera root extract. 1663–1673. https://doi.org/10.1007/s00262-013-1470-2

[65] Slee EA, O'Connor DJ, Lu X. To die or not to die: how does p53 decide? Oncogene. 2004 Apr 12;23(16):2809-18. doi: 10.1038/sj.onc.1207516. PMID: 15077144.

[66] Sørlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M. B., Rijn, M. Van De, Jeffrey, S. S., Thorsen, T., Quist, H., Matese, J. C., Brown, P. O., Botstein, D., Eystein, P., & Børresen-dale, A. (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. 98(19). https://doi.org/10.1073/pnas.191367098

[67] Sporn, M. B. (1991). Perspectivesin CancerResearch Carcinogenesis and Cancer: Different Perspectives on the Same Disease1. 6215–6218.

[68] Srinivasan S, Ranga RS, Burikhanov R, Han SS, C. D. (n.d.). Par-4-Dependent Apoptosis by the Dietary Compound Withaferin A in Prostate Cancer Cells. Cancer Res 2007;67:246–53. https://doi.org/https://doi.org/10.1158/0008-5472.CAN-06-2430

[69] Srivastav, S., Fatima, M., & Mondal, A. C. (2017). Important medicinal herbs in Parkinson's disease pharmacotherapy. Biomedicine and Pharmacotherapy, 92, 856–863. https://doi.org/10.1016/j.biopha.2017.05.137

[70] Stan SD, Zeng Y, S. S. (2008). Ayurvedic Medicine Constituent Withaferin A Causes G2 and M Phase Cell Cycle Arrest in Human Breast Cancer Cells. Nutr Cancer 2008;60(Suppl 1):51–60. [PubMed: 19003581], 60, 51–60.

[71] Stan, S. D., Hahm, E. R., Warin, R., & Singh, S. V. (2008). Withaferin A causes FOXO3a- and Bim-dependent apoptosis and inhibits growth of human breast cancer cells in vivo. Cancer Research, 68(18), 7661–7669. https://doi.org/10.1158/0008-5472.CAN-08-1510

[72] Suman K Samanta , Joomin Lee , Eun-Ryeong Hahm, S. V. S. (2018). Peptidyl-prolyl cis/trans isomerase Pin1 regulates withaferin A-mediated cell cycle arrest in human breast cancer cells. Mol Carcinog. 2018 Jul;57(7):936-946. Doi: 10.1002/Mc.22814. Epub 2018 Apr 16, 57(7), 936–946. https://doi.org/10.1002/mc.22814

[73] Szarc, K., Beeck, K. Op De, Ratman, D., Wouters, A., Beck, I. M., Declerck, K., Heyninck, K., Fransen, E., Bracke, M., Bosscher, K. De, Lardon, F., Camp, G. Van, & Berghe, W. Vanden. (2014). Pharmacological Levels of Withaferin A (Withania somnifera) Trigger Clinically Relevant Anticancer Effects Specific to Triple Negative Breast Cancer Cells. 9(2). https://doi.org/10.1371/journal.pone.0087850

[74] Tandon, N., & Yadav, S. S. (2020). Safety and clinical effectiveness of Withania somnifera (Linn) Dunal root in human ailments. Journal of Ethnopharmacology, 255(August 2019), 112768. https://doi.org/10.1016/j.jep.2020.112768

[75] Thaiparambil, J. T., Bender, L., Ganesh, T., Kline, E., Patel, P., Liu, Y., Tighiouart, M., Vertino, P. M., Harvey, R. D., Garcia, A., & Marcus, A. I. (2011). Withaferin A inhibits breast cancer invasion and metastasis at sub-cytotoxic doses by inducing vimentin disassembly and serine 56 phosphorylation. International Journal of Cancer, 129(11), 2744–2755. https://doi.org/10.1002/ijc.25938

[76] Thompson, H. J., Adlakha, H., Thompson, H. J., & Adlakha, H. (1991). Dose-responsive Induction of Mammary Gland Carcinomas by the Intraperitoneal Injection of 1-Methyl-1-nitrosourea Dose-responsive Induction of Mammary Gland Carcinomas by the Intraperitoneal Injection of 1-Methyl-l-nitrosourea1. 3411–3415.

[77] Vyas, A. R., & Singh, S. V. (2014). Molecular targets and mechanisms of cancer prevention and treatment by Withaferin A, a naturally occurring steroidal lactone. AAPS Journal, 16(1), 1–10. https://doi.org/10.1208/s12248-013-9531-1

[78] Wang, F., Zhao, J., Bai, J., Gao, K., Cui, D., Chen, Y., & Song, Y. (2019). Liquid chromatography-tandem mass spectrometry to assess the pharmacokinetics and tissue distribution of withaferin A in rats ☆. Journal of Chromatography B, 1122–1123(April), 90–95. https://doi.org/10.1016/j.jchromb.2019.05.016

[79] Wankhede, S., Langade, D., Joshi, K., Sinha, S. R., & Bhattacharyya, S. (2015). Examining the effect of Withania somnifera supplementation on muscle strength and recovery: A randomized controlled trial. Journal of the International Society of Sports Nutrition, 12(1), 1–11. https://doi.org/10.1186/s12970-015-0104-9

[80] Xiaokang Liu , Yu Li 1, Qiang Ma, Y. W. A. L. S. (2019). Withaferin-A Inhibits Growth of Drug-Resistant Breast Carcinoma by Inducing Apoptosis and Autophagy, Endogenous Reactive Oxygen Species (ROS) Production, and Inhibition of Cell Migration and Nuclear Factor kappa B (Nf-κB)/Mammalian Target of Rapamycin (m-. Med Sci Monit. 2019 Sep 12;25:6855–6863. Doi: 10.12659/MSM.916931, 25, 6855–6863. https://doi.org/10.12659/MSM.916931

[81] Yang, H., Shi, G., & Dou, Q. P. (2007). The tumor proteasome is a primary target for the natural anticancer compound Withaferin A isolated from "Indian Winter Cherry." Molecular Pharmacology, 71(2), 426–437. https://doi.org/10.1124/mol.106.030015

[82] Yang, Z., Garcia, A., Xu, S., Powell, D. R., Vertino, P. M., Singh, S., & Marcus, I. (2013). Withania somnifera Root Extract Inhibits Mammary Cancer Metastasis and Epithelial to Mesenchymal Transition. 8(9), 1–12. https://doi.org/10.1371/journal.pone.0075069

[83] Yoneyama T, Arai MA, Sadhu SK, Ahmed F, Ishibashi M. Hedgehog inhibitors from Withania somnifera. Bioorg Med Chem Lett 2015;25:3541–4. [PubMed: 26169123]

[84] Yu F, Li J, Chen H, Fu J, Ray S, Huang S, et al. Kruppel-like factor 4 (KLF4) is required for maintenance of breast cancer stem cells and for cell migration and invasion. Oncogene 2011;30:2161–72. [PubMed: 21242971] 87.

[85] Zhang, X., Mukerji, R., Samadi, A. K., & Cohen, M. S. (2011). Down-regulation of estrogen receptor-alpha and rearranged during transfection tyrosine kinase is associated with withaferin a-induced apoptosis in MCF-7 breast cancer cells.

[86] Zhang, X., Timmermann, B., Samadi, A. K., & Cohen, M. S. (2012). Withaferin A Induces Proteasome-Dependent Degradation of Breast Cancer Susceptibility Gene 1 and Heat Shock Factor 1 Proteins in Breast Cancer Cells. 2012. https://doi.org/10.5402/2012/707586